N°d'ordre : 3791

UNIVERSITE DES SCIENCES ET TECHNOLOGIES DE LILLE

UFR DE BIOLOGIE

UNIVERSITE LIBRE DE BRUXELLES

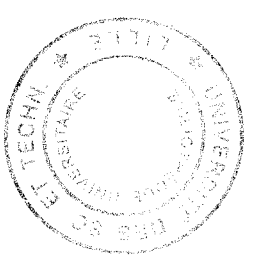
THESE

Présentée pour obtenir le grade de
Docteur de l'Université de Lille I et
Docteur de l'Université Libre de Bruxelles
Spécialité : Biologie et Génétique des Populations
Ecole doctorale : Sciences de la matière, du Rayonnement
et de l'Environnement

Characterisation of zinc and cadmium tolerance in Arabidopsis halleri

présentée et soutenue publiquement par

Glenda WILLEMS



Soutenue le 13 mars 2006 devant le jury composé de :

M. Cuguen Joël	Prof. Université de Lille1 (F)	Président
Mme Outi Savolainen	Prof. Université de Oulu (FIN)	Rapporteur
M. Steve Barnes	Project Leader Molecular Screening (B)	Rapporteur
M. Henk Schat	Dr. Université Libre d'Amsterdam (NL)	Examinateur
Mme Nathalie Verbruggen	Prof. Université libre de Bruxelles (B)	Co-directeur de thèse
M. Pierre Saumitou-Laprade	Dr. Université de Lille1 (F)	Directeur de thèse

REMERCIEMENTS

J'ai commencé ma thèse en octobre 2002 au laboratoire de Génétique et Evolution des Populations Végétales (GEPV) de l'Université de Lille où j'ai effectué la majorité de mon travail de recherche, et au laboratoire de Physiologie et Génétique Moléculaire des Plantes (LPGMP) de l'Université Libre de Bruxelles.

Je tiens à remercier tout d'abord Prof. Nathalie Verbruggen du laboratoire LPGMP. Si j'ai fait cette thèse de doctorat c'est un peu grace à elle. Elle a su me convaincre, elle m'a conseillé et aidé à faire les premières démarches pour dénicher un poste de doctorante au sein du réseau européen *Metalhome*. Je me souviens encore de ma première visite au laboratoire GEPV et ma première réunion avec Pierre Saumitou-Laprade. J'étais impressionnée par ce grand laboratoire, toutes ces machines et le sujet de thèse m'avait l'air fort compliqué. Heureusement, Pierre était là pour répondre à toutes mes questions, pour me conseiller et aider. Il m'a donné la possibilité de suivre les formations nécessaires pour que je sache mener à bien toutes les expériences effectuées pendant ces trois ans et demi. Il faut absolument que je mentionne sa grande disponibilité à tout moment lorsque des problèmes inattendus surgissaient dans le cadre du travail ou autre. Avec tout cela il m'a transmis son enthousiasme pour l'existence des phénoménès étranges qui sont la tolérance aux métaux lourds et l'hyperaccumulation des métaux lourds chez *Arabidopsis halleri* ; c'est pour cela que j'ai effectué ma thèse avec plaisir et que j'en garderai un très bon souvenir.

Au sein du laboratoire GEPV, je tiens également à remercier plus particulièrement Cécile et Anne. Elles m'ont beaucoup aidé dans mon travail, elles étaient toujours là pour résoudre mes petits problèmes techniques et Cécile connaissait toujours une astuce, qui m'avait forcément échappé, pour faciliter les manips. J'ai pu tirer beaucoup de leçons de la rigueur de travail irreprochable des deux filles. Même en dehors du travail, nous avons passé des moments très agréables que je conserverai précisieusement dans ma mémoire. Il faut également que je remercie les autres membres

du groupe des « Métaux Lourds » du laboratoire GEPV. Ils étaient là quand j'avais besoin d'eux pour préparer ou terminer mes expériences en serre, qui souvent comptaient un beau nombre de plantes, ou tout simplement pour discuter et me conseiller sur mon travail. Un grand merci aussi à l'équipe de la serre. Robert toujours très efficace dans son travail, de bonne humeur et parlant avec un accènt auquel j'ai dû m'habituer un peu avant de pouvoir le déchiffrer, racontant des blagues les unes plus drôles que les autres, mais qui ont toujours mis une bonne ambiance lorsqu'il fallait encore faire des boutures. Eric qui ramenait un peu d'ambiance belge dans la serre, vu qu'il est un grand fan de la radio belge. Je lui ai appris ce qu'étaient des conducteurs phantômes ou encore que ce n'était pas rare de voir des chiens ou parfois même des vâches errant sur les autoroutes belges. Puis Claire, c'était la bonne humeur assurée, le petit soleil et l'arrivée de BOB, le poisson rouge. Je n'oublierai pas ces bons moments passés à la serre qui souvent me faisaient du bien: l'ambiance y était plus relaxe qu'au labo et, on y était tranquille et puis j'aimais bien travailler avec les plantes, le terreau etc. Je tiens encore à remercier les deux thésards Stéphane et Maxime. Nous avons commencé notre thèse pratiquement au même moment au laboratoire et nous avions donc ce petit truc en commun. Surtout à la fin de la thèse, pendant la rédaction, c'était bien d'avoir des copains qui se trouvaient parfois dans le même désespoir. C'était toujours sympathique de pouvoir discuter et rigoler avec vous et d'avoir vos petits mots de soutien et d'encouragement. Je remercie aussi tous les autres collègues du laboratoire GEPV. A commencer évidemment par Joel Cuguen, le directeur du labo qui m'a permis de faire ma thèse au sein de son laboratoire. Il a aussi montré beaucoup de patience avec mon ordinateur, qui s'était mis un matin à émettre pleins de virus sur tout le campus universitaire... Je n'aurai pas assez de place pour mentionner tout le monde du GEPV, mais j'ai beaucoup apprécié le temps que j'ai passé avec vous tous.

Je remercie également le laboratoire LPGMP de l'ULB: Nathalie Verbruggen pour les multiples discussions que nous avons eues ensemble, les conseils qu'elle m'a donnés et sa confiance dans mon travail, Pietro pour les bons moments passés ensemble dans les serres entre autres, Adrian, Mikael, Alban et Nancy qui ont fait des visites au laboratoire des petits moments de plaisir, détente et rigolade.

I also keep very nice souvenirs of the multiple meetings to which I participated, within or outside the RTN Metalhome. I had the chance to meet very interesting persons, Henk Schat, Mark Aarts, Sebastien Thomine, Stephan Clemens, Ute Krämer, Andrew Smith and the young researchers, Sangitta, Kerstin, Marc, Ronald, Alex and Antoine. I apologize for the people that I might have forgot to mention, and want to thank you all for the great moments we spent together during these meetings. J'ai en outre fait la connaissance de Marie et Isabelle du laboratoire de Biochimie et Physiologie Moléculaire des Plantes de l'Unversité de Montpellier, et de Geraldine Sarret du laboratoire de Geophysique Interne et Tectonophysique de l'Université de Grenoble. Je me souviens de notre séjour à Daresbury au Royaume-Uni, pour faire des expériences dans le synchrotron. Cet endroit m'a beaucoup impressionné et sera fort probablement une expérience unique pour moi !

En dernier lieu, je tiens à remercier mes parents, mes grands-parents, ma sœur et son mari qui m'ont soutenu et qui m'ont permis d'arriver jusque-là. Un grand merci à Luc qui m'a soutenu, encouragé, donné de la confiance et a su m'apporter du réconfort et de la consolation dans des moments difficiles. Puis, les copains qui en passant par notre nouvelle demeure à Beauvechain m'ont permis de me changer les idées pendant ces six derniers mois de rédaction et donc de beaucoup de stress. Même s'ils n'ont pas toujours dû comprendre ce que je pouvais bien faire tous les jours derrière cet ordinateur, j'ai toujours beaucoup apprécié leurs visites qui constituaient des petits moments de détente.

Allez (c'est pour vous, Maxime et Pierre, cette petite note belge !), je vous laisse maintenant à la lecture de ma thèse…

TABLE OF CONTENTS

Introduction

1 HEAVY METAL TOLERANCE, AN ADAPTIVE RESPONSE TO HEAVY METAL POLLUTED SITES

1.1 Prologue

Evolutionary biologists have a long-standing interest in the genetic basis of biological diversity or, natural variation between individuals or populations, either belonging to the same or different species (Stearns and Hoekstra 2000; Olsen and Purugganan 2002). Some of this variation has shown to be adaptive in nature, which means that the variation is heritable, that it evolved by natural selection to function better, through improved survival and reproductive performance, in a given context (Stearns and Hoekstra 2000; Schlötterer 2002; Bijlsma and Loeschcke 2005). Understanding the genetic architecture of, eventually adaptive, traits is a complex issue and implies, according to Mackay (2001; 2004) the knowledge of (a) the genes underlying the trait and leading to the trait phenotype, (b) the loci that are responsible for the naturally occurring variation in the trait, (c) the homozygous, heterozygous, epistatic and pleiotropic effects of the alleles in a wide range of environments, (d) the molecular basis of the allelic variation and finally, (e) the evolutionary forces responsible for maintaining the genetic variation in nature for the trait.

Long before the discovery of the basic principles of the modern genetics, scientists attempted even then by the establishment of theoretical models to explain the occurrence of adaptive traits in nature and the action through which natural selection produced adaptation. Darwin for instance, argued in his theory of evolution that "natural selection can act only by taking advantage of slight successive variations; she can never take a leap, but must advance by the shortest and slowest steps" (Darwin 1859). The discovery of the principles of inheritance by Mendel vigorously challenged the Darwinian view through which natural selection was believed to act to produce

adaptation. An important step forward in the understanding of the action through which natural selection could produce adaptation was achieved with the development of infinitesimal theory (Fisher 1930). In this theory Fisher fused successfully the Darwinian view of adaptation and the Mendelian laws of inheritance. Fisher stated in his model that the response to selection would occur through random mutations, i.e. random with respect to their impact on the phenotype. Most mutations would have a relatively small effect, since mutations of large effect would tend to be deleterious and rapidly eliminated from the population by natural selection. According to Fisher's theory adaptive traits would be governed by an innumerable number of genes inherited following the Mendelian laws, each unlinked to each other, each having no epistatic interactions with the others, and each having a minute effect on the character (Fisher 1930; Farrall 2004; Orr 2005). Supported by evidence from biological analyses for the involvement in adaptation of few genes with relatively large effects, some modifications of Fisher's model were introduced first by Kimura (1983) and secondly by Orr (1998). Finally, an exponential model was obtained for predicting the genetic basis of adaptive traits. This model stated that the distribution of factors fixed during adaptation should be roughly exponential and that the first factors fixed often can be fairly large (Orr 1998; Farrall 2004; Orr 2005).

Heavy metal tolerance was firmly established in the literature as one of the best examples of adaptation (Bradshaw 1970; Antonovics *et al.* 1971; Baker 1987), and could represent an excellent model system in the study of the genetic bases of an adaptive trait in plants. In the present work we investigate the genetic architecture of heavy metal tolerance in the zinc and cadmium tolerant species *Arabidopsis halleri* ssp. *halleri*. In this thesis, we will attempt to provide an answer on the following questions:

(a) How many loci are involved in zinc and cadmium tolerance in *A. halleri* ssp. *halleri*?

(b) How do they contribute to zinc and cadmium tolerance in *A. halleri* ssp. *halleri*?

(c) How do they interact with each other?

(d) Are zinc and cadmium tolerance under an independent genetic control?

(e) What are the genes potentially involved in zinc and cadmium tolerance in *A. halleri* ssp. *halleri*?

1.2 Heavy metal tolerance

Heavy metal tolerance, shared by a relatively small number of plant species, the majority of which belongs to the Leguminosae and Gramineae families (Antonovics *et al.* 1971; Baker 1987), evolved in response to the high selective pressures exerted by the enrichment in heavy metals (see Annexes **BOX 1**) of heavy metal polluted soils. Heavy metal contaminated soils have been reported worldwide either occurring naturally as in serpentine soils, which are abundant in nickel (Ni), chrome (Cr) and cobalt (Co), or as a consequence of industrial activities (mining, the aerial fallout from smelters or vehicle exhausts, the toxic run-off of galvanized structures or copper roofs, sewage sludges and the use of fungicides, pesticides or disinfectants) that contributed to the enrichment of soils in copper (Cu), zinc (Zn), lead (Pb), Ni, Co, mercury (Hg) and cadmium (Cd) (Antonovics *et al.* 1971; Macnair 1993; Saxena *et al.* 1999).

Metal tolerance was first described by Pratt in 1934, who observed that seed of *Melandrium silvestre* (later referred to as *Silene vulgaris*) from a Cu mine grew far better than seed of *M. silvestre* from an uncontaminated site in soils showing higher Cu concentrations. Thus, plants originating from metallicolous sites exhibited an increased capacity to cope with high metal concentrations, probably through a shift in the dose-response curve (see Annexes **BOX 2**) (Macnair 1993; Hagemeyer 1999), enabling them to grow and reproduce on heavy metal contaminated soils (Bradshaw 1970; Antonovics *et al.* 1971; Macnair 1993). Metal tolerance was initially believed to be restricted to those populations growing on metalliferous sites and was related to the ability of normally non-tolerant species to evolve tolerant races through the presence of the appropriate genetic information (Antonovics *et al.* 1971). However, some plant species, for instance *A. halleri*, have been reported to exhibit heavy metal tolerance throughout the species range (Macnair *et al.* 1999; Bert *et al.* 2000). Contrary to those species in which heavy metal tolerance is restricted to metallicolous populations, and in which

consequently the evolution of the adaptation can be easily dated (as old as the heavy metal pollution of the site), the evolution of constitutional or constitutive metal tolerance remains elusive.

Because industrial sites are generally contaminated by a combination of heavy metals, metallophyte species often exhibit tolerance to various metals rather than to just one (Antonovics *et al.* 1971). For instance, the metallicolous site on which the *A. halleri* individual used in the present work was collected, showed high Zn/Pb/Cd concentrations (Van Rossum *et al.* 2004). Consistent with this multiple contamination the *A. halleri* individual exhibited tolerance to both Zn (see Chapter 4) and Cd (see Chapter 5) (Bert *et al.* 2000; Bert *et al.* 2003; Courbot *et al.* submitted; Willems *et al.* submitted). Natural selection most probably resulted in the development of specific mechanisms conferring tolerance to those metals present at high concentrations in the soil, i.e. functional tolerances (Antonovics *et al.* 1971; Schat and Vooijs 1997). Functional tolerances however, have been reported in some cases to produce non-functional or co-tolerances, which are believed to be due to similar biochemical properties of different heavy metals (Schat and Vooijs 1997; Tilstone and Macnair 1997).

Two contrasting behaviours have been distinguished among metal tolerant plants, more precisely exclusion and hyperaccumulation. Most metallophytes behave as excluders, i.e. they restrict metal uptake and accumulation either by preventing metals from entering the plant (true exclusion) or by restricting the translocation of metals to the shoots (shoot exclusion) (Baker 1981). In rare cases metal tolerant species have been observed to accumulate metals to extremely high and toxic amounts in their aboveground parts (Baker 1981). This phenomenon, called hyperaccumulation (Brooks *et al.* 1977), has been reported for less than 0.2% of the angiosperms or approximately 400 species (Baker 1981; Brooks 1998; Macnair 2003). Almost 75% of the hyperaccumulators concentrate high levels of Ni (Brooks 1998). They are generally found on serpentine soils, being highly enriched in this metal. Zn hyperaccumulators constitute the second largest group of the known hyperaccumulators. The *A. halleri* species for instance possesses the ability to accumulate high Zn levels in its leaves

(Macnair *et al.* 1999; Bert *et al.* 2000; Macnair 2002; Macnair 2003). In addition, *A. halleri* is one of the two species reported to date to hyperaccumulate Cd (Bert *et al.* 2002; Bert *et al.* 2003). Hyperaccumulation is believed to be an adaptive trait similarly to heavy metal tolerance (Assunçao *et al.* 2003; Macnair 2003), although its adaptive function is difficult to conceive and at least much less evident than the adaptive function of metal tolerance. Globally, five hypotheses have been suggested regarding the adaptive value of hyperaccumulation: hyperaccumulation functions (a) to increase the metal tolerance of the plant, (b) to increase the drought resistance of leaves, (c) to increase the pathogen or herbivore defence, (d) to suppress other competitors by the creation of a zone of toxic soil, (e) as an inadvertent consequence of high-affinity uptake of other elements that may be scarce in mineralised substrates (Boyd and Martens 1992). The pathogen or herbivore defence hypothesis is currently the most studied (Boyd and Martens 1992; Boyd and Martens 2002; Noret *et al.* 2005).

1.3 References

Antonovics, J., Bradshaw, A. D., and Turner, R. G., 1971 Heavy metal tolerance in plants. *Advances in Ecological Research* **7:** 1-85.

Assunçao, A. G. L., Schat, H., and Aarts, M. G. M., 2003 *Thlaspi caerulescens*, an attractive model species to study heavy metal hyperaccumulation in plants. *New Phytologist* **159:** 351-360.

Baker, A. J. M., 1981 Accumulators and excluders - strategies in the response of plants to heavy metals. *Journal of Plant Nutrition* **3:** 643-654.

Baker, A. J. M., 1987 Metal tolerance. *New Phytologist* **106:** 93-111.

Bert, V., Macnair, M. R., de Laguerie, P., Saumitou-Laprade, P., and Petit, D., 2000 Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri* (Brassicaceae). *New Phytologist* **146:** 225-233.

Bert, V., Bonnin, I., Saumitou-Laprade, P., de Laguerie, P., and Petit, D., 2002 Do *Arabidopsis halleri* from nonmetallicolous populations accumulate zinc and cadmium more effectively than those from metallicolous populations? *New Phytologist* **155:** 47-57.

Bert, V., Meerts, P., Saumitou-Laprade, P., Salis, P., Gruber, W., and Verbruggen, N., 2003 Genetic basis of Cd tolerance and hyperaccumulation in *Arabidopsis halleri*. *Plant and Soil* **249:** 9-18.

Bijlsma, R., and Loeschcke, V., 2005 Environmental stress, adaptation and evolution: an overview. *Journal of Evolutionary Biology* **18:** 744-749.

Boyd, R. S., and Martens, S. N., 1992 The *raison d'être* for metal hyperaccumulation by plants, pp. 279-289 in *The vegetation of ultramafic (serpentine) soils: proceedings of the First International Conference on Serpentine Ecology*, edited by A. J. M. Baker, J. Proctor and R. D. Reeves. Intercept Limited, U. K.

Boyd, R. S., and Martens, S. N., 2002 The defensive role of Ni hyperaccumulation by plants: a field experiment. *American Journal of Botany* **89:** 998-1003.

Bradshaw, A. D., 1970 Plants and industrial waste. *Transactions of the Botanical Society of Edinburgh* **41:** 71-84.

Brooks, R. R., Lee, J., Reeves, R. D., and Jaffré, T., 1977 Detection of nickeliferous rocks by analysis of herbarium specimes of indicator plants. *Journal of Geochemical Exploration* **7:** 49-57.

Brooks, R. R., 1998 *Plants that hyperaccumulate heavy metals.* Cab International, Wallingford.

Courbot, M., Willems, G., Motte, P., Arfvidsson, S., Saumitou-Laprade, P., and Verbruggen, N., submitted to *Plant Journal* The major QTL for cadmium tolerance in *Arabidopsis halleri* co-localizes with *HMA4*, a gene encoding a Heavy Metal ATPase.

Darwin, C. R., 1859 *The origin of species.* J. Murray, London.

Farrall, M., 2004 Quantitative genetic variation: a post-modern view. *Human Molecular Genetics* **13:** R1-R7.

Fisher, R. A., 1930 *The genetical theory of natural selection.* Oxford University Press, Oxford.

Hagemeyer, J., 1999 Ecophysiology of plant growth under heavy metal stress in *Phytoremediation of heavy metal contaminated and polluted soils*, edited by M. N. V. Prasad and J. Hagemeyer. Springer, Berlin.

Kimura, M., 1983 *The neutral theory of molecular evolution.* Cambridge University Press, Cambridge.

Mackay, T. F., 2001 The genetic architecture of quantitative traits. *Annual Review of Genetics* **35:** 303-339.

Mackay, T. F., 2004 The genetic architecture of quantitative traits: lessons from Drosophila. *Current Opinion in Genetics and Development* **14:** 253-257.

Macnair, M. R., 1993 Tansley review No. 49: The genetics of metal tolerance in vascular plants. *New Phytologist* **124:** 541-559.

Macnair, M. R., Bert, V., Huitson, S. B., Saumitou-Laprade, P., and Petit, D., 1999 Zinc tolerance and hyperaccumulation are genetically independent characters. *Proceedings of the Royal Society of London, Series B: Biological Sciences* **266:** 2175-2179.

Macnair, M. R., 2002 Within and between population genetic variation for zinc accumulation in *Arabidopsis halleri*. *New Phytologist* **155:** 59-66.

Macnair, M. R., 2003 The hyperaccumulation of metals by plants. *Advances in Botanical Research* **40:** 64-106.

Noret, N., Meerts, P., Tolra, R., Poschenrieder, C., Barcelo, J., and Escarre, J., 2005 Palatability of *Thlaspi caerulescens* for snails: influence of zinc and glucosinolates. *New Phytologist* **165:** 763-772.

Olsen, K. M., and Purugganan, M. D., 2002 Plant population genomics, linkage disequilibrium mapping, and the genetics of adaptation, pp. in *Plant Adaptation: Molecular Genetics and Ecology.*, edited by J. W. Q.C.B. Cronk, R. H. Ree and I.E.P. Taylor, Vancouver, British Columbia, Canada.

Orr, H. A., 1998 The population genetics of adaptation: the distribution of factors fixed during adaptive evolution. *Evolution* **52:** 935-949.

Orr, H. A., 2005 The genetic theory of adaptation: a brief history. *Nature Reviews Genetics* **6:** 119-127.

Saxena, P. K., KrishnaRaj, S., Dan, T., Perras, M. R., and Vettakkorumakankav, N. N., 1999 Phytoremediation of heavy metal contaminated and polluted soils in *Heavy metal stress in plants. From molecules to ecosystems.*, edited by M. N. V. Prasad and J. Hagemeyer. Springer, Berlin.

Schat, H., and Vooijs, R., 1997 Multiple tolerance and co-tolerance to heavy metals in *Silene vulgaris*: a co-segregation analysis. *New Phytologist* **136:** 489-496.

Schlötterer, C., 2002 Towards a molecular characterization of adaptation in local populations. *Current Opinion in Genetics and Development* **12:** 683-687.

Stearns, S. C., and Hoekstra, R. F., 2000 *Evolution: an introduction.* Oxford University Press, New York.

Tilstone, G. H., and Macnair, M. R., 1997 Nickel tolerance and copper - nickel co-tolerance in *Mimulus guttatus* from copper mine and serpentine habitats. *Plant and Soil* **191:** 173-193.

Van Rossum, F., Bonnin, I., Fenart, S., Pauwels, M., Petit, D., and Saumitou-Laprade, P., 2004 Spatial genetic structure within a metallicolous population of *Arabidopsis halleri*, a clonal, self-incompatible and heavy-metal-tolerant species. *Molecular Ecology* **13:** 2959-2967.

Willems, G., Godé, C., Dräger, D., Courbot, M., Verbruggen, N., and Saumitou-Laprade, P., submitted to *Genetics* Quantitative Trait Loci mapping of zinc tolerance in the metallophyte *Arabidopsis halleri* ssp. *halleri.*

2 STATE OF THE ART OF THE GENETIC AND PHYSIOLOGICAL BASES INVOLVED IN HEAVY METAL TOLERANCE AND HYPERACCUMULATION

2.1 Strategies deployed in the study of heavy metal tolerance and hyperaccumulation

The phenomenon of heavy metal tolerance was established in the literature as one of the best examples of local adaptation and microevolution, providing insights in several aspects of the evolutionary process (Antonovics *et al.* 1971). Additionally, since the past twenty years the scientific community showed renewed interest in heavy metal tolerance and hyperaccumulation because of the potential use of metal tolerant and hyperaccumulator species in phytoremediation (Salt *et al.* 1998; Krämer 2005b). This green technology for the clean up or stabilisation of industrial polluted sites (Brooks 1998; Macnair *et al.* 2000) highly stimulated the research in the genetic and physiological bases underlying these traits. However, it was the development of the appropriate tools that enabled scientists finally to conduct this research.

On the one hand, tolerance tests were established providing a means for measuring and quantifying tolerance (Wilkins 1978; Macnair 1983; Schat and Ten Bookum 1992) (see *2.3.1*), which was until then impossible. Indeed, the early reports of tolerance and non-tolerance were merely based on the presence or absence of growth on contaminated soils in natural or controlled conditions (Bradshaw 1970; Antonovics *et al.* 1971). The study of the genetic determinism of heavy metal tolerance could now be performed through the analysis of its segregation in progenies obtained through crossing individuals with contrasting metal tolerance phenotypes (see *2.3.2*). Moreover, the co-

segregation of tolerance to various metals could be analysed and provided a means to distinguish between multiple tolerance (metal specific mechanisms) and co-tolerance (pleiotropic mechanisms) (Schat and Vooijs 1997; Tilstone and Macnair 1997) (see *2.3.2*). Additionally, crosses implicating metal tolerant and hyperaccumulator species enabled the co-segregation of tolerance and hyperaccumulation; the dependent versus independent relationship between both traits could be as such established (Macnair *et al.* 1999; Assunçao *et al.* 2003c; Frerot *et al.* 2005) (see *2.3.4*). On the other hand, the development of a wide range of molecular tools initially restricted to the currently used model species highly promoted the study of the physiological bases underlying heavy metal tolerance and hyperaccumulation (see *2.4.1* and *2.4.2*). To date this research can be performed even in non-model species, though exhibiting the traits of interest (see **2.2**) (Assunçao *et al.* 2003a; Feder and Mitchell-Olds 2003; Weber *et al.* 2004).

2.2 *Arabidopsis halleri*, a model species in the study of heavy metal tolerance and hyperaccumulation

In the study of heavy metal tolerance and hyperaccumulation the need for additional model species was prompted because of the absence of both traits in the currently used model species, like *A. thaliana*. To date, *A. halleri* has been considered an excellent model system to study heavy metal tolerance and hyperaccumulation (Becher *et al.* 2004; Weber *et al.* 2004; Willems *et al.* submitted). The *A. halleri* species belongs to the family of the Brassicaceae and more precisely to the Arabidopsis genus, which contains about ten species that are native to Eurasia, North Africa and North America (Mitchell-Olds 2001) (Figure 2.1).

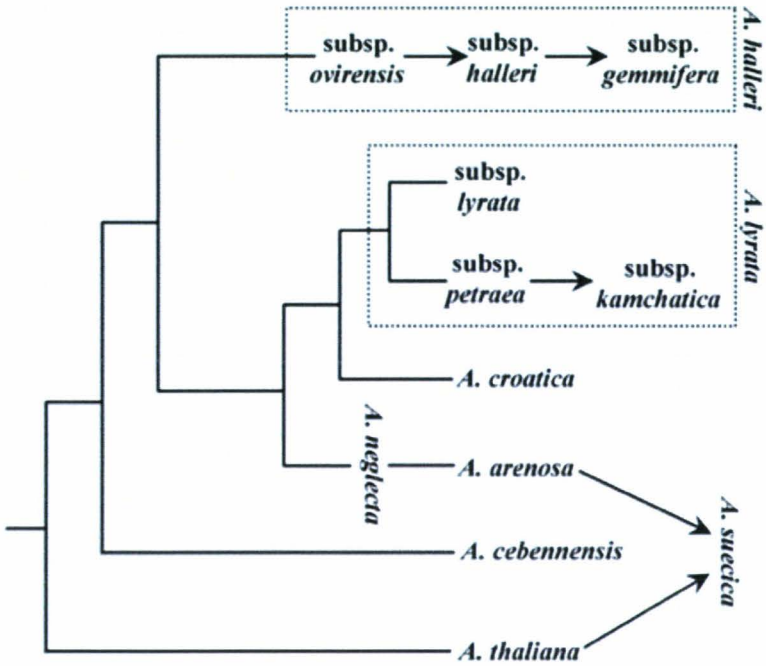


FIGURE 2.1 The Arabidopsis genus, phylogenetic tree (according to Al-Shebaz and O'Kane 2002).

A. halleri (n = 8) is a perennial, self-incompatible and insect-pollinated species. *A. halleri* plants produce large numbers of seeds and show in addition a high clonal ability (Mitchell-Olds 2001; Van Rossum *et al.* 2004). Within the *A. halleri* species three subspecies can be distinguished (Al-Shehbaz and O'Kane 2002). The *halleri* subspecies[1] is a pseudometallophyte, distributed in Europe among continental and mountainous areas, occurring on acidic, fresh, oligotrophic and heavy metal contaminated soils (Bert *et al.* 2002) (Figure 2.2) (Figure 2.3). *A. thaliana*, the most studied species within the Arabidopsis genus is distinct from the other Arabidopsis species with regard to its reproductive system and chromosome number. Whereas the latter are predominantly allogamous, *A. thaliana* is a largely autogamous species. In addition, the divergence of *A. thaliana* from its closest wild relatives *A. halleri* and *Arabidopsis lyrata*, estimated at approximately 5.8 Million Years, involved a reduction from eight to five chromosomes (Koch *et al.* 2001; Mitchell-Olds 2001).

[1] For simplification we will henceforth adopt the term *A. halleri* to refer to its metallophyte subspecies *A. h. halleri*.

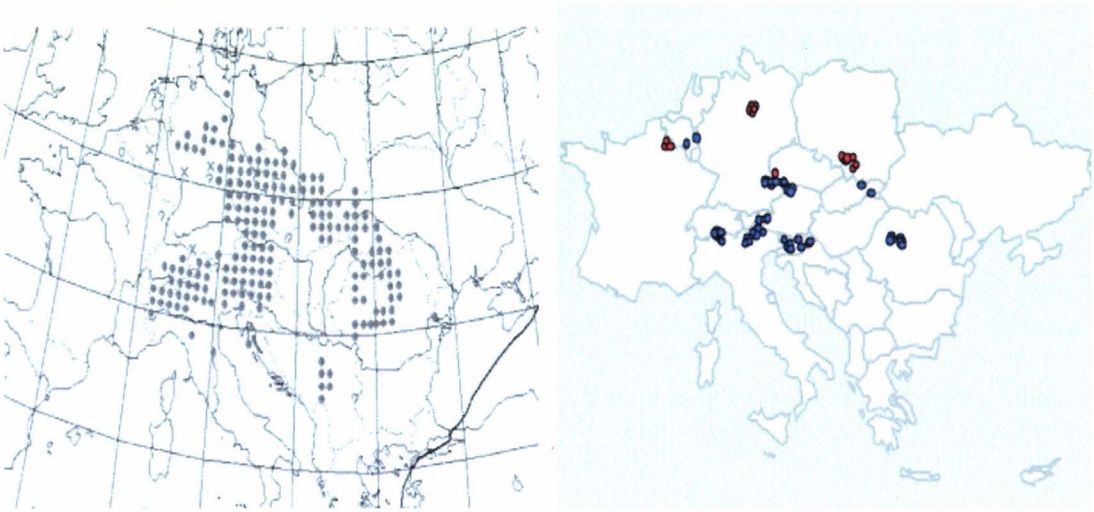


FIGURE 2.2 Distribution area of *A. halleri* in Europe.

Right panel: *A. halleri* distribution map, according to *Flora Europea*
Left panel: metallicolous (red circles) and non-metallicolous (bleu circles) *A. halleri* populations, collected in 1999 (Pauwels *et al.* 2005) and 2003 (Pauwels *et al.* in prep) in the northern and southern part of the species distribution range. These populations were used to study the population structure of the metallophyte species and to establish the origin of the metallicolous *A. halleri* populations (Pauwels *et al.* 2005).

The close proximity of *A. halleri* and *A. thaliana* as well as the high DNA identity in the coding regions between both species (90 to 95%) (Weber *et al.* 2004) involves that the wide range of "-omic" tools that have been currently developed for the model species *A. thaliana* through the knowledge of its genome, can be readily transferred to *A. halleri* (Mitchell-Olds 2001; Feder and Mitchell-Olds 2003). This enhances importantly the investigation of the molecular bases of heavy metal tolerance and hyperaccumulation in this species. Moreover, the study of other non-model but closely related Arabidopsis species, like *A. lyrata* for instance, has been recently initiated. To date genetic linkage maps are available for the *A. lyrata* subspecies *petraea* (Kuittinen *et al.* 2004) and *lyrata* (Yogeeswaran *et al.* 2005) and the sequencing of the *A. lyrata* ssp. *petraea* genome has been started this year (http://www.jgi.doe.gov/sequencing/why/CSP2006/AlyrataCrubella.html). These research projects constitute precious information resources for *A. halleri*, the closest relative of *A. lyrata*.

FIGURE 2.3 *A. halleri* individuals on the Zn/Pb/Cd polluted site located in proximity of the Zn smelter Umicor situated in Auby (North of France) (Van Rossum *et al.* 2004). An *A. halleri* individual of this population was used in the present work.

Similarly to *A. halleri*, the metal tolerant and hyperaccumulator species *Thlaspi caerulescens* (2n = 14) has been currently brought about as an attractive model species in the study of heavy metal tolerance and hyperaccumulation (Assunçao *et al.* 2003a). Contrary to *A. halleri T. caerulescens* is fully self-compatible, which might constitute an advantage compared to *A. halleri* for carrying out genetic analyses. Nevertheless, because of the high clonal ability of *A. halleri* (Van Rossum *et al.* 2004) replicates can be easily obtained for this species. *T. caerulescens* is less related to *A. thaliana* than *A.*

halleri (88% DNA identity within the coding regions) (Assunçao *et al.* 2003a); this implies a more difficult transfer of the *A. thaliana* tools to *T. caerulescens*.

2.3 The genetic bases of heavy metal tolerance

2.3.1 The measurement of tolerance

Initially, tolerance was measured by the calculation of a tolerance index, corresponding to the length of root growth in toxic solutions expressed as a percentage of growth in the control solution (Gregory and Bradshaw 1964; Jowett 1964) or, to the ratio of root growth in a metal concentration over root growth in a control solution (Wilkins 1978). These tolerance tests revealed a dominance relationship between tolerance and sensitivity, although variation in the direction of dominance was observed between crosses. Clear-cut Mendelian segregations were rarely found, and this was interpreted as evidence for polygenic control. However, the validity of the tolerance index and consequently, of the results obtained were vigorously challenged because this tolerance measure was believed to exhibit an inherently high level of statistical noise (Macnair 1983; Schat and Ten Bookum 1992; Macnair 1993). Indeed, the tolerance index was a quotient of two variables with presumably differently skewed probability distributions and additionally, affected by innate variation of root growth, and thus by other genes than those that were supposed to govern tolerance (Macnair 1983; Schat and Ten Bookum 1992). In order to provide more accurate estimates of tolerance Macnair (1983) introduced the single concentration test, in which tolerance was evaluated by the ability of cuttings to produce roots at a certain fixed metal concentration. Contrary to the tolerance index, this tolerance test gave clear-cut Mendelian segregations, even though the results were found to be highly dependent on the metal concentration applied in the test (Schat and Ten Bookum 1992; Macnair 1993). To overcome the limitations inherent to the single concentrations test, the multiple concentration test was adopted, in which clones were exposed to different metal concentrations and tolerance corresponded to the concentration at which growth was reduced to 50% of control root growth (Macnair 1993). However, this tolerance test was relatively time-consuming

because of the series of metal concentrations at which tolerance had to be estimated as well as the generation time necessary for the production of clonal replicates (Schat and Ten Bookum 1992; Macnair 1993). Moreover, some plant species were found to be particularly inappropriate for vegetative propagation. In order to avoid the cloning of individual plants an alternative type of the multiple concentration test, i.e. the sequential exposure test (Schat and Ten Bookum 1992) was established, in which "each individual plant was exposed to a test solution in which the metal concentration was increased in time in a stepwise manner" and the tolerance level corresponded to the lowest concentration at which root growth was completely inhibited (Schat and Ten Bookum 1992). To date, multiple concentration tests might be considered the most suitable for addressing the genetic determinism of heavy metal tolerance. These tolerance tests have been widely used in genetic analyses, though upon some slight modifications according to the species used in the test (Bert *et al.* 2000; Assunçao *et al.* 2003b; Bert *et al.* 2003; Willems *et al.* submitted).

2.3.2 Segregation analyses of heavy metal tolerance

The genetic determinism of tolerance to various metals in different metallophytes investigated either through single concentration or multiple concentration tests, showed to be consistent with simple genetic models composed of one or two major genes with additive gene action. Through the development of intraspecific crosses involving tolerant and sensitive individuals evidence was provided for those simple genetic models for instance in *Mimulus guttatus* (one major gene) (Macnair 1983) and *S. vulgaris* (one or two major gene(s) depending on the population) for Cu tolerance (Schat and Ten Bookum 1992; Schat *et al.* 1993), in *Holcus lanatus* (one major gene) (Macnair 1993) and *Agrostis capillaris* (one major gene) (Watkins and Macnair 1991) for As tolerance and in *S. vulgaris* for Zn tolerance (two major genes) (Schat *et al.* 1996) . In all cases, tolerance was found to be a dominant character. In addition to the major gene(s) conferring metal tolerance, one or a few modifier genes, supposed to be hypostatic to the major tolerance gene(s) were suggested to explain the quantitative variations in tolerance among the tolerant individuals (Watkins and Macnair 1991; Schat *et al.* 1996; Macnair *et al.* 2000). In *A. halleri* the genetic analysis

of Zn tolerance was initially impeded by the presence of this trait throughout the species range (Macnair *et al.* 1999; Bert *et al.* 2000). However, this major handicap was successfully overcome by the development of crosses between *A. halleri* and its close non-tolerant relative *A. l. petraea*, which ensured the segregation of Zn tolerance (Macnair *et al.* 1999). In the F2 progeny developed on one *A. l. petraea* x *A. halleri* cross the segregation of Zn tolerance was interpreted as the effect of one or a few major gene(s) (Macnair *et al.* 1999; Macnair *et al.* 2000). Using a similar crossing scheme, Bert *et al.* (2003) analysed the segregation of Cd tolerance. The segregation ratios for Cd tolerance in an *A. halleri* x *A. l. petraea* backcross progeny indicated the presence of two or three major genes with additive effect (Bert *et al.* 2003).

With the development of tolerance tests a means was also provided for investigating tolerance to various metals and moreover, for distinguishing between multiple and co-tolerances (Schat and Vooijs 1997; Tilstone and Macnair 1997; Bert *et al.* 2003). In analysing crosses between metallicolous *S. vulgaris* individuals, Zn tolerance showed to co-segregate with tolerance to either Ni or Co, whereas an independent segregation was reported for Zn and Cu tolerance (Schat *et al.* 1996; Schat and Vooijs 1997). Based on these results Cu and Zn tolerance were supposed to be under the control of different genes (multiple tolerances) whereas through pleiotropy Zn tolerance was expected to confer tolerance to Ni and Co (co-tolerances) (Schat *et al.* 1996; Schat and Vooijs 1997). In *A. halleri*, a positive and significant correlation was observed between Zn and Cd tolerance upon the analysis o of an *A. halleri* x *A. l. petraea* backcross progeny, even though large variations in Zn tolerance were reported for the more Cd-tolerant individuals (Bert *et al.* 2003). Both metal tolerances were believed to be under the control of common major genes or linked genes; nonpleiotropic genes or modifiers were probably involved too (Bert *et al.* 2003).

2.3.3 Co-segregation of heavy metal tolerance and hyperaccumulation

The close association of heavy metal tolerance and hyperaccumulation in plant species such as *A. halleri* and *T. caerulescens* does not necessarily mean that these characters are under an identical genetic control. In *A. halleri* for instance, the co-

segregation of Zn tolerance and hyperaccumulation was analysed in an *A. halleri* x *A. l. petraea* F2 progeny (Macnair *et al.* 1999). The independent segregation of both traits was interpreted as the existence of different loci governing Zn tolerance and hyperaccumulation (Macnair *et al.* 1999). Contrary to *A. halleri*, variation for Ni/Cd/Zn tolerance and hyperaccumulation was reported among different *T. caerulescens* accessions (Assunçao *et al.* 2003c; Frerot *et al.* 2005).

2.4 The physiological bases of heavy metal tolerance and hyperaccumulation

All higher plants developed a tightly knit network of mechanisms, i.e. the metal homeostasis network (Figure 2.4) to maintain essential metals in the cytosol at concentrations within their physiological limits and nonessential ones at low non-toxic cytosolic concentrations (Clemens 2001; Hall 2002). The ability to cope with varying concentrations of heavy metals in their growth environment is consequently common to all higher plants, though this so-called "basic" metal tolerance is highly different from the metal tolerance characterising metallophyte species. Indeed, metallophyte species developed the ability to withstand metal concentrations that are several magnitudes higher than those that are concerned in "basic" metal tolerance and the term "hypertolerance" has therefore been used in some cases to refer to the tolerance acquired by metallophyte species (Clemens 2001). However, since we presume that "basic" metal tolerance reflects metal homeostasis rather than metal tolerance, we adopt the term "tolerance" to designate the metal tolerance exhibited by metallophyte species.

The metal homeostasis network common to all higher plants has been focussed on in a large range of studies investigating the physiological bases of metal tolerance and hyperaccumulation, because these traits are believed to have evolved through adaptations of processes involved in metal homeostasis (Clemens 2001; Clemens *et al.* 2002; Hall 2002). Two strategies have been adopted consisting either in the characterisation of metal homeostasis processes in metal tolerant, eventually hyperaccumulating species (Figure 2.4) (see *2.4.1*), or in the identification of genes

potentially involved in cellular metal homeostasis in the non-tolerant, though well-known species *A. thaliana* as well as in the metal tolerant, and hyperaccumulating species *A. halleri* and *T. caerulescens* (see *2.4.2*). We emphasize more particularly on genes encoding metal transporters, since these constitute important components of the metal homeostasis network. They are for instance involved in the transport of metals from the soil solution into the root and in the distribution of these metals throughout the whole plant, crossing both cellular and organellar membranes (Mäser *et al.* 2001; Hall and Williams 2003; Hanikenne *et al.* 2005). To date, four metal transporter families have been intensively studied in *A. thaliana*: the Zrt- (zinc-regulated transporter), Irt-(iron-egulated transporter) like proteins, the natural resistance-associated macrophage proteins, the P-type ATPases and the Cation Diffusion Facilitators. In addition, several *A. halleri* and *T. caerulescens* homologous genes encoding members of these metal transporter families have been identified and/or functionally characterised.

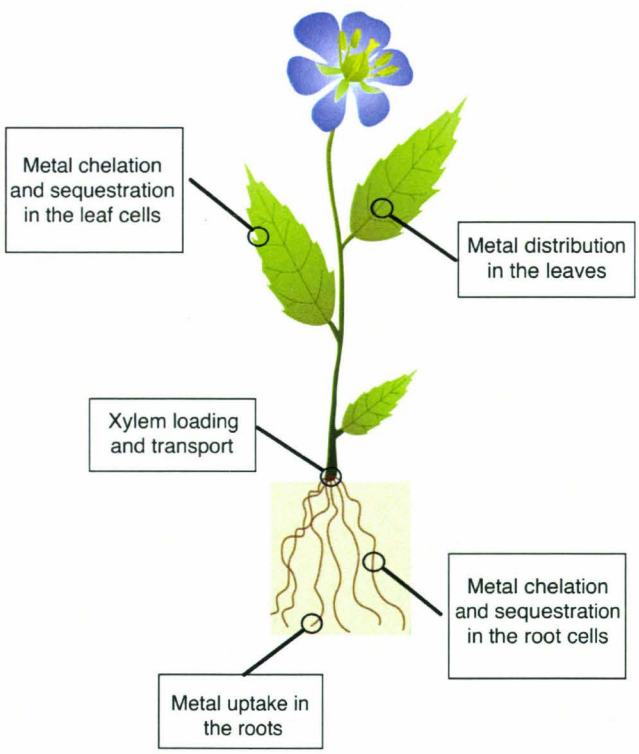


FIGURE 2.4 Metal homeostasis processes proposed to be involved in metal tolerance and hyperaccumulation in plants, according to Clemens *et al.* 2001.

In the following pages (pages 25 to 37) we present an extensive overview of the physiological bases of heavy metal tolerance and hyperaccumulation. The metal homeostasis processes and the metal transporter genes potentially involved in these traits have been summarised in Table 2.1 and Table 2.2 respectively. This overview offers us the opportunity to draw a clear image of the current state of the art of the physiological bases of heavy metal tolerance and hyperaccumulation in metallophytes generally, and in *A. halleri* particularly (see **2.5**). Some of the elements presented in the following pages have moreover been used in our study; we analysed for instance the implication in adaptive metal tolerance in *A. halleri* of several metal homeostasis genes, which were presented here.

2.4.1 Characterisation of metal homeostasis processes involved in heavy metal tolerance and hyperaccumulation

2.4.1.1 Metal uptake in the roots

As (V) tolerance in the grasses *H. lanatus, A. capillaris* and *Deschampsia cespitosa* is to date the clearest example of reduced uptake characterising true excluder metallophytes as an adapted tolerance mechanism in response to arsenic toxicity and could be attributed to the suppression of the high affinity phosphate/arsenate uptake system (Meharg 1994; Bleeker 2004). Through the reduction of As (V) influx it was believed that the plant could detoxify the metal by mechanisms (chelation, vacuolar sequestration, As (V) reduction) common to all higher plants (Meharg and Macnair 1992; Meharg 1994; Hartley-Whitaker *et al.* 2001; Bleeker 2004). In contrary, in sensitive populations these detoxification mechanisms were expected to be inhibited more rapidly because of the increased As (V) influx in these populations. Reduced metal uptake was furthermore reported in the roots of Cu-tolerant *S. vulgaris* individuals when compared to Cu-sensitive ones under identical growth conditions (De Vos *et al.* 1991). In the former modifications of the root cell plasma membrane were supposed to prevent Cu from binding the plasma membrane, resulting as such in a reduced Cu uptake (De Vos *et al.* 1991). Moreover, an enhanced Cu efflux across the root cell plasma membrane was observed in *S. vulgaris* tolerant ecotypes (van Hoof *et al.* 2001b).

Contrary to the reduced metal uptake characterising metal tolerant excluders, a significantly higher root Zn influx was observed in the hyperaccumulator *T. caerulescens* when compared to *Thlaspi arvense,* a closely related non-tolerant and non-accumulator species (Lasat *et al.* 1996; Lasat *et al.* 2000). The kinetics of root Zn influx indicated similar affinities of the transporter systems in both species; the difference in Zn influx was probably due to a higher density of Zn transporters in the root cell plasma membrane of the *T. caerulescens* species (Lasat *et al.* 1996; Lasat *et al.* 2000; Pence *et al.* 2000). However, despite a greater Zn influx into the *T. caerulescens* roots more Zn was accumulated in roots of *T. arvense.* The authors suggested that this could be due to an enhanced Zn translocation to the shoots in *T. caerulescens* or to an increased sequestration in *T. arvense* root cell vacuoles, rendering the metal unavailable for translocation to the shoots (Lasat *et al.* 2000).

2.4.1.2 Metal chelation and sequestration in the roots

In all higher plants previous to their sequestration (in the root and leaf cells), metals are chelated in the cytosol by high-affinity ligands (phytochelatins (PCs), metallothioneins (MTs), organic and amino acids) to counteract their high reactivity as well as to increase their solubility. Among these chelators PCs and MTs are expected to route metals predominantly to root sequestration, whereas organic and amino acids are supposed to route metals primarily to the xylem (see 2.4.1.3) (Clemens *et al.* 2002). PCs are peptides with the general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, where n = 2 to 10. They are synthesized enzymatically using glutathione (GSH; γ-Glu-Cys-Gly) as the substrate (Cobbett and Goldsbrough 2002); the enzyme PC synthase catalyses the transpeptidation of the γ-Glu-Cys moiety of GSH either onto a second GSH molecule to form $PC_{(n=2)}$ or onto a PC molecule to produce a $PC_{(n+1)}$ oligomer. Consistent with the implication of PCs in heavy metal detoxification, PC synthesis is induced in vivo and in vitro by several metals (Cobbett and Goldsbrough 2002). In contradiction to PCs, MTs, cysteine-rich cytoplasmic metal-binding proteins, are not enzymatically synthesized, but genetically encoded (Zhou and Goldsbrough 1995).

Although PCs were expected to play a major role in adaptive metal tolerances, evidence for their implication in the detoxification of heavy metals in metal tolerant species could only be provided for the As (V)-tolerant *H. lanatus*. In the roots of As-tolerant populations, PC synthesis was reported to be 15- to 20-fold higher than in the roots of As-sensitive ones when grown in equal metal concentrations (Hartley-Whitaker *et al.* 2001; Schat *et al.* 2002). The increased PC synthesis in As-tolerant populations is presumably involved in adaptive As tolerance though the major determinant is certainly As (V) uptake (Meharg and Macnair 1992; Hartley-Whitaker *et al.* 2001). PC synthesis in the roots has furthermore been studied in relationship to Zn, Cd, Ni and Cu tolerance in *S. vulgaris* (Harmens *et al.* 1993; de Knecht *et al.* 1995; Schat *et al.* 2002) as well as to Zn, Cd and Ni tolerance in *T. caerulescens* (Schat *et al.* 2002). However, in none of the studies evidence could be provided for an implication of PCs in adaptive metal tolerance. On the one hand PC synthesis was found to be higher in sensitive populations compared to tolerant ones; on the other hand the tolerant individuals did not show an increased sensitivity upon inhibition of PC synthesis under high metal concentrations (Schat *et al.* 2002).

A role for metallothioneins in adaptive metal tolerance was initially suggested upon their functional characterisation in non-tolerant plant species, such as *A. thaliana*. The *A. thaliana* genes *MT1* and *MT2*, encoding metallothioneins conferred increased tolerance to Cu and Cd in yeast (Zhou and Goldsbrough 1995) and *AtMT2* expression could moreover be significantly and positively correlated to basic Cu tolerance surveyed in 10 *A. thaliana* ecotypes (Murphy and Taiz 1995). In Cu-(hyper)tolerant *S. vulgaris* populations *MT2* was significantly higher expressed, in the roots as well as in the leaves, compared to Cu-sensitive populations, probably related to *MT2*-gene amplification in the former (van Hoof *et al.* 2001a). Additionally, *MT2* expression co-segregated with Cu tolerance in families derived from crosses between moderately tolerant F3 plants and suggested a role of *MT2* in adaptive Cu tolerance, although only in a genetic tolerance background (van Hoof *et al.* 2001a).

With the exception of those heavy metal tolerant species showing exclusion of the metal through avoidance (for instance As (V) tolerant species), excluders store

metals predominantly in the root cells. Enhanced vacuolar sequestration capacities were suggested to play a role in naturally selected Zn tolerance in *S. vulgaris* (Chardonnens *et al.* 1999), upon the observation of increased tonoplast transport in the root cells of Zn-tolerant *S. vulgaris* populations compared to Zn-sensitive ones (Verkleij *et al.* 1998; Chardonnens *et al.* 1999), as well as in homozygous Zn-sensitive and tolerant F3 plants, originating from crosses between Zn-tolerant and sensitive *S. vulgaris* ecotypes (Chardonnens *et al.* 1999).

2.4.1.3 Xylem loading and transport

In metal tolerant hyperaccumulator species xylem loading and transport might be involved in adaptive metal tolerance and/or hyperaccumulation. In *T. caerulescens* for instance, Zn accumulation in the xylem sap was found to be significantly higher than in the metal sensitive and non-accumulating *T. arvense* species (Lasat *et al.* 1996; Lasat *et al.* 1998; Lasat *et al.* 2000). Zn tolerance and hyperaccumulation in *T. caerulescens* might consequently be attributed at least partially, to increased translocation of this metal from the roots to the shoots (Lasat *et al.* 1996; Lasat *et al.* 1998; Lasat *et al.* 2000). However, this is apparently far from being a general rule for metal tolerant hyperaccumulator species, as indicated by the non-significant difference in xylem transport of Ni between the Ni-tolerant hyperaccumulator *T. goesingense* and *T. arvense* (Krämer *et al.* 1997). Furthermore, in the Ni-tolerant hyperaccumulator species *Alyssum montanum* the presence of high amounts of exogenously supplied histidine resulted in a 50-fold increase in the rate of Ni transport into the xylem, suggesting a potential role for metal chelation in Ni tolerance and/or hyperaccumulation at least in *A. montanum* (Krämer *et al.* 1996).

2.4.1.4 Metal distribution in the leaves

In metal tolerant hyperaccumulator species metals are preferentially accumulated in the leaves. In *A. halleri* for instance the shoot to root ratios for Zn were consistently higher than 1, while in the non-tolerant non-accumulator species *A. lyrata* and *A. thaliana* the opposite pattern was observed (Bert *et al.* 2000; Bert *et al.* 2003). A distinct pattern of metal distribution in the foliar tissues was observed in both tolerant hyperaccumulator species *T. caerulescens* and *A. halleri*. Whereas Zn was stored

preferentially in the leaf epidermal tissue in the former (Küpper *et al.* 1999), this metal was predominantly accumulated in the leaf mesophyll tissue in the latter (Küpper *et al.* 2000). In *T. caerulescens*, the preferential accumulation of Zn in the epidermal tissue may be implicated in Zn tolerance through the protection of the mesophyll from the build-up and the toxicity of high Zn concentrations, allowing as such to maintain the functionality of the mesophyll cells over a wide range of foliar Zn concentrations (Küpper *et al.* 1999). High Zn concentrations were also reported to occur in a ring-shaped narrow region at the base of trichomes, although their function in metal storage or exudation is still unclear (Küpper *et al.* 2000; Sarret *et al.* 2002).

2.4.1.5 Metal chelation and sequestration in the leaves

Similarly to metal tolerant excluder species, enhanced vacuolar sequestration, although in the leaf cells rather than in the roots, might play a key role in adaptive metal tolerance (and/or hyperaccumulation) in metal tolerant hyperaccumulator species. In the Ni-tolerant and hyperaccumulator *T. goesingense* for instance, approximately two-fold more Ni was accumulated in the leaf cell vacuoles when compared to *T. arvense* even though leaf and protoplast concentrations were equivalent in both species (Krämer *et al.* 2000). The metal was furthermore found to be stored under the form of Ni-citrate and Ni-histidine complexes in *T. goesingense* and *T. arvense* respectively, which supported the efficient vacuolar storage capacity of Ni in the hyperaccumulator species. Indeed, Ni-citrate complexes were known to be very stable under the pH conditions within the vacuolar compartment, whereas the chelation of metals by histidine was likely to occur in a neutral pH environment such as the cytoplasm (Krämer *et al.* 2000). In the metallophyte *A. halleri* Zn was found predominantly under the form of Zn-malate complexes in its aerial parts, in the non-tolerant non-accumulator *A. lyrata*, Zn-phosphate complexes were identified (Sarret *et al.* 2002). Interestingly, although malate was present in similar quantities in both species, the ability of forming Zn-malate complexes was apparently specific to the hyperaccumulator species (Sarret *et al.* 2002) and might eventually be involved in Zn tolerance and/or hyperaccumulation.

TABLE 2.1 Metal homeostasis processes proposed to be involved in metal tolerance and/or hyperaccumulation

Metal homeostasis process	Metal	Species	Reference
Metal uptake in the roots			
reduced uptake[a]	As	*H. lanatus*	Meharg and Macnair 2002
reduced uptake[b]	Cu	*S. vulgaris*	De Vos *et al.* 1991
increased efflux	Cu	*S. vulgaris*	Van Hoof *et al.* 2001
increased influx	Zn	*T. caerulescens*	Lasat *et al.* 2000
Metal chelation and sequestration in the roots			
phytochelatins	As	*H. lanatus*	Hartley-Whitaker *et al.* 2001
metallothioneins	Cu	*S. vulgaris*	Van Hoof *et al.* 2001
enhanced vacuolar sequestration	Zn	*S. vulgaris*	Chardonnens *et al.* 1999
Xylem loading and transport			
increased metal loading in xylem	Zn	*T. caerulescens*	Lasat *et al.* 2000
chelation by histidine	Ni	*T. goesingense*	Krämer *et al.* 1996
Metal distribution in the leaves			
storage in the epidermis	Zn	*T. caerulescens*	Küpper *et al.* 1999
storage in the mesophyll	Zn	*A. halleri*	Küpper *et al.* 2000
Metal chelation and sequestration in the leaves			
enhanced vacuolar sequestration	Ni	*T. goesingense*	Krämer *et al.* 2000
chelation by malate	Zn	*A. halleri*	Sarret *et al.* 2002

[a] through the suppression of the high affinity arsenate/phosphate uptake system
[b] through plasma membrane modifications

2.4.2 Characterisation of genes underlying metal homeostasis: metal transporter families

2.4.2.1 The Zrt-, Irt-like proteins

The Zrt-, Irt-like proteins (ZIP) are capable of transporting a variety of cations, including Cd, Fe, Mn and Zn (Guerinot and Eide 1999; Guerinot 2000). In *A. thaliana*, the ZIP family counts one iron (Fe)-regulated (IRT1) and 14 Zn-regulated transporters (Mäser *et al.* 2001). The ZIP gene *IRT1* was identified in *A. thaliana* through the screening for cDNA clones that were able to restore Fe limited growth in a yeast strain

defective for Fe uptake (Eide *et al.* 1996). Expression of *AtIRT1* in yeast revealed furthermore that IRT1 was able of transporting Cd, Co, Mn and Zn in addition to Fe (Eide *et al.* 1996). In *A. thaliana*, high transcript levels of *IRT1* were observed in the roots of Fe-deficient plants. The lack of *IRT1* expression in the shoots as well as in the roots under Fe-sufficient conditions suggests a role of *AtIRT1* in the uptake of Fe from the rhizosphere across the plasma membrane in the root epidermis (Eide *et al.* 1996). Similarly to *AtIRT1*, the *A. thaliana* ZIP genes *ZIP1*, *ZIP2* and *ZIP3* were isolated by functional expression in the mutant yeast strains (Guerinot 2000). *AtZIP1* and *AtZIP3* are probably involved in Zn uptake in the roots as might be suggested by their expression in the roots of Zn-deficient plants and their potential plasma membrane targeting sequence (Grotz *et al.* 1998). Unlike the other ZIP genes, *AtZIP2* shows affinity for Cu and Cd in addition to Zn at a similar degree. Moreover, its expression was observed in neither the shoots nor the roots of Zn-deficient or sufficient plants and a function for ZIP2 in Zn uptake could consequently not be suggested (Grotz *et al.* 1998). *AtZIP4*, identified through a DNA sequence database analysis, showed a response to Zn deficiency as indicated by its expression in both roots and shoots under Zn-deficient conditions and this transporter was predicted to localise in the chloroplast membrane (Grotz *et al.* 1998; Guerinot and Eide 1999; Guerinot 2000). A role for AtZIP4 in Zn transport within or between cells was proposed (Grotz *et al.* 1998; Guerinot and Eide 1999; Guerinot 2000).

In *T. caerulescens*, the *AtZIP4* ortholog *ZNT1* was identified through a yeast complementation experiment. The Zn transporter TcZNT1 was believed to be responsible for the enhanced Zn influx in the roots of *T. caerulescens* when compared to *T. arvense*, because of the similar kinetic properties of ZNT1 and Zn transport in the roots of the hyperaccumulator species (Lasat *et al.* 2000; Pence *et al.* 2000; Lasat *et al.* 1996; Lasat *et al.* 1998). Additionally, *TcZNT1* was significantly higher expressed than in the non-accumulator *T. arvense*. Assunçao *et al.* (2001) confirmed high *ZNT1* expression in three *T. caerulescens* accessions exhibiting different Zn tolerance levels. A role for ZNT1 in Zn hyperaccumulation rather than tolerance was suggested (Assunçao *et al.* 2001). Contrary to *TcZNT1*, *ZNT1* expression in *T. arvense* was regulated by the external Zn concentrations. A downregulation of *ZNT1* expression in *T.*

caerulescens was however observed but only after a prolonged exposure to Zn (Lasat *et al.* 2000; Pence *et al.* 2000).

Microarray analyses conducted on *A. halleri* and *A. thaliana* using *A. thaliana* GeneChips representing approximately 8300 genes, resulted in the identification of several *A. halleri* orthologs of *A. thaliana* metal homeostasis genes (Becher *et al.* 2004; Weber *et al.* 2004). The *AtZIP9* ortholog for instance was reported to be 43-fold higher expressed in *A. halleri* roots when compared to *A. thaliana* upon exposure to low Zn concentrations (0.8 μM) (Weber *et al.* 2004). In addition, the *AhZIP9* transcript level was downregulated upon treatment with high Zn concentrations (Weber *et al.* 2004). In *A. halleri* and *A. thaliana* shoots, the *A. halleri AtZIP6* ortholog was reported to be 23- to 24-fold higher expressed than *AtZIP6* under both low (1 μM) or high (300 μM and 100 μM for *A. halleri* and *A. thaliana* respectively) $ZnSO_4$ concentrations (Becher *et al.* 2004). Under these Zn conditions *AhZIP6* was also higher expressed than *AtZIP6* in the roots, although the difference in expression between both genes was less important (4- to 9-fold) (Becher *et al.* 2004). Contrary to the differential expression of *ZIP6* in the roots and shoots and of *ZIP9* in the roots in *A. halleri* and *A. thaliana*, similar expression was observed for *ZIP1* in the roots and the shoots and for *ZIP4* in the shoots of both species (Becher *et al.* 2004). In the roots however, under low Zn concentrations (1 μM $ZnSO_4$) *AhZIP4* was significantly higher expressed than *AtZIP4* (Becher *et al.* 2004). In contrast to the downregulation of *AtZIP4* under high Zn concentrations, the expression of *AhZIP4* remained high even under Zn-replete conditions (Becher *et al.* 2004). As observed in the expression analyses conducted on the ZIP orthologs in the hyperaccumulator species *T. caerulescens* (Lasat *et al.* 2000; Pence *et al.* 2000; Assunçao *et al.* 2001) and *A. halleri* (Becher *et al.* 2004; Weber *et al.* 2004) extremely enhanced expression of certain metal homeostasis genes under low metal supply might constitute a constitutive property in metal hyperaccumulators (Weber *et al.* 2004). Moreover, in the hyperaccumulator species the regulation of the expression of those genes appeared to require Zn concentrations several magnitudes higher than in ordinary, non-accumulator species in order to be activated. Apparently, differences exist in the sensing of the metal status in hyperaccumulators relative to non-hyperaccumulator species (Weber *et al.* 2004).

2.4.2.2 The natural resistance-associated macrophage proteins

The natural resistance-associated macrophage proteins (NRAMPs) are involved in metal transport in a wide range of organisms, including bacteria, fungi, plants and animals. In *A. thaliana* six NRAMPs have been identified, which group into two classes based on sequence homologies. One class is represented by *AtNRAMP1* and *AtNRAMP6* and the other includes *AtNRAMP2* to *5* (Thomine *et al.* 2000; Mäser *et al.* 2001; Hall and Williams 2003). First evidence for the implication of AtNRAMP1, AtNRAMP3 and AtNRAMP4 in Fe and Mn transport was provided through the efficient complementation of a yeast mutant defective in Fe and Mn uptake (Curie *et al.* 2000). A role for NRAMPs in Fe transport was additionally supported by the observation in *A. thaliana* of an upregulated expression of *AtNRAMP1*, *AtNRAMP3* and *AtNRAMP4* in the roots as well as in the shoots in the case of *AtNRAMP4* under Fe starvation (Curie *et al.* 2000; Thomine *et al.* 2000). In addition, a function of *AtNRAMP3* in Mn and Zn accumulation upon Fe starvation and Cd sensitivity was proposed through the mobilisation of Fe, Cd and other metals from the vacuolar compartment. This was deduced from the vacuolar localisation of AtNRAMP3 and its expression in the vascular tissue of the roots and the shoots, as well as from the analysis of *AtNRAMP3*-overexpressing lines, which showed increased Cd sensitivity and reduced Mn and Zn accumulation upon Fe starvation (Thomine *et al.* 2000). Consistent with the latter observations, *A. thaliana* mutant lines lacking a functional AtNRAMP3 transporter exhibited increased Cd resistance and higher Mn and Zn content when compared to the wild type (Thomine *et al.* 2000).

In *A. halleri* the *AtNRAMP3* ortholog was identified in a microarray analysis conducted on *A. thaliana* and *A. halleri* because of its significantly higher expression (8.4-fold) in the roots when compared to *AtNRAMP3* upon exposure to low Zn concentrations (0.8 μM) (Weber *et al.* 2004). In the shoots, *AhNRAMP3* was also higher expressed than *AtNRAMP3*, although at lower levels than in the roots (Weber *et al.* 2004). Similarly to the regulation of the ZIP genes, *AhNRAMP3* shoot and root expression were not affected by Zn treatment, while *AtNRAMP3* expression was downregulated in the roots and upregulated in the shoots under high Zn concentrations

(Weber *et al.* 2004). The authors suggested that the stronger expression of *AhNRAMP3* might result in a reduced Zn sequestration in the root cells, because of a higher mobilization of this metal from the vacuole and consequently, in a higher root to shoot translocation of Zn (Weber *et al.* 2004).

2.4.2.3 The P-type ATPases

P-type ATPases form a large family of transporter proteins, which hydrolyse ATP for transporting a broad range of small cations, and possibly phospholipids, across cell membranes; they have been found in bacteria, fungi, yeast, animals and plants (Axelsen and Palmgren 2001; Hanikenne *et al.* 2005). In *A. thaliana* a total of 46 genes encoding P-type ATPases have been identified (Baxter *et al.* 2003). The P-type ATPases cluster in five subfamilies according to sequence and functional similarities. The P_{1B} subfamily is involved in the transport of heavy metals and these ATPases are therefore currently referred to as heavy-metal P-type ATPases (HMAs) (Baxter *et al.* 2003; Bernard *et al.* 2004; Papoyan and Kochian 2004). Eight HMAs have been identified in *A. thaliana* clustering in two groups according to substrate specificity and sequence homology. AtHMA5, AtHMA6, AtHMA7 and AtHMA8 are involved in the transport of monovalent cations, such as Cu^{+} and silver (Ag^{+}) and potentially in metal homeostasis as was recently demonstrated for AthHMA5 (Andrès-Colas *et al.* 2006). The predominant expression of this transporter in the roots and the strong and specific induction of *AtHMA5* expression by Cu in the whole plant as well as the increased sensitivity to Cu of *A. thaliana* mutant lines carrying a defective HMA5 protein suggested a role for *AtHMA5* in Cu detoxification in roots (Andrès-Colas *et al.* 2006). The second group includes AtHMA1, AtHMA2, AtHMA3 and AtHMA4 and they were suggested to transport cations, such as Zn^{2+}, Cd^{2+}, Co^{2+} and Pb^{2+} given their close relationship to divalent cation transporters from prokaryotes (Hall and Williams 2003; Hanikenne *et al.* 2005). Evidence for a function of *AtHMA2* and *AtHMA4* in Zn homeostasis was provided through the efficient complementation of an *Escherichia coli* mutant defective in an endogenous Zn pump (Mills *et al.* 2003) as well as the analysis of *A. thaliana hma2hma4* double mutants showing extreme Zn deficiency, higher root and less shoot accumulation than the wild type upon exposure to identical Zn concentrations (Hussain *et al.* 2004). Additionally, the ectopic overexpression of

AtHMA4 resulted in increased Cd and Zn resistance and increased translocation of these metals (Verret *et al.* 2004). *AtHMA2* and *AtHMA4* were expressed predominantly in the vascular bundle of both the roots and the shoots and localised to the plasma membrane (Hussain *et al.* 2004; Verret *et al.* 2004). Based on these findings, a function in Zn and Cd homeostasis and more precisely in xylem loading and unloading and in the remobilisation of Zn and Cd through the phloem was proposed for AtHMA2 and AtHMA4 by mediating the transport of these metals across the plasma membrane (Hussain *et al.* 2004; Verret *et al.* 2004; Verret *et al.* 2005). AtHMA3 was recently proposed to be involved in Cd homeostasis and presumably in vacuolar sequestration given the localisation of this metal pump in the tonoplast (Gravot *et al.* 2004).

The *T. caerulescens AtHMA4* ortholog has been intensively studied in relationship to Cd homeostasis following its characterisation through a yeast complementation screen of a *T. caerulescens* cDNA library to identify cDNAs conferring Cd tolerance (Bernard *et al.* 2004; Papoyan and Kochian 2004). *TcHMA4* was significantly higher expressed in the roots compared to the shoots and this was confirmed in three *T. caerulescens* populations differing in Cd tolerance and accumulation (Bernard *et al.* 2004; Papoyan and Kochian 2004). Similarly to AtHMA4, TcHMA4 was proposed to be implicated in the root to shoot translocation of Cd and probably Zn (Bernard *et al.* 2004; Papoyan and Kochian 2004). The *A. halleri AtHMA3* ortholog was identified through comparative microarray analysis conducted on the shoots of *A. halleri* and *A. thaliana* plants and was 220- to 270-fold higher expressed in the metallophyte upon exposure to low (1 μM) and high (300 μM and 100 μM for *A. halleri* and *A. thaliana* respectively) $ZnSO_4$ concentrations and an upregulation of the *AhHMA3* expression was observed under high Zn concentrations (Becher *et al.* 2004). In the roots *AhHMA3* transcript levels were comparable to those observed in *A. thaliana* under low Zn conditions and an increased expression was reported under high Zn conditions in both species though *AhHMA3* reached a higher expression than *AtHMA3* under the latter conditions. The highest expression levels however, were unambiguously observed in the shoots. The authors suggested that the immediate detoxification of Zn in the shoots, as expected from the abundance of *AhHMA3* mRNAs, could generate a metal sink in the shoots and therefore, play a major role in Zn hyperaccumulation in *A.*

halleri (Becher *et al.* 2004). The ATPase HMA4 was recently investigated in *A. halleri* with regard to its role in Cd, and eventually Zn homeostasis (Courbot *et al.* submitted). An expression analysis conducted on *AhHMA4* indicated a consistently higher expression in Cd-tolerant *A. halleri* x *A. l. petraea* BC1 individuals when compared to Cd-sensitive ones and the same pattern was observed in an *A. halleri* individual when compared to a Cd-sensitive *A. l. petraea* individual. Moreover, the expression of *AhHMA4* was approximately eightfold higher in the roots than in the shoots in all Cd-tolerant individuals. Similarly to AtHMA4, the *AhHMA4* protein localised in the plasma membrane and evidence for the Zn and Cd transport function of AhHMA4 was provided through its expression in yeast strains (Courbot *et al.* submitted). A function for AhHMA4 in the protection of the roots cells predominantly, against the build-up of toxic cytosolic Zn and Cd concentrations was suggested (Courbot *et al.* submitted).

2.4.2.4 The cation diffusion facilitator protein family

The cation diffusion facilitator (CDF) protein family includes metal transporters of the cations Zn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+} and Mn^{2+}. They catalyse the efflux of these metal ions from the cytoplasm to the outside of the cell or into a subcellular compartment. In the *A. thaliana* genome sequence eight genes were found encoding probably members of the CDF family (Mäser *et al.* 2001). The first CDF protein identified and intensively studied in *A. thaliana* (Kobae *et al.* 2004; Desbrosses-Fonrouge *et al.* 2005; Krämer 2005a) was the metal tolerance protein 1 (MTP1), formerly known as ZAT (van der Zaal *et al.* 1999). By the analysis of the subcellular localisation of AtMTP1 as well as of *A. thaliana* mutant lines either overexpressing or silencing *AtMTP1*, a role in Zn detoxification was proposed through the transport of Zn across the vacuolar membrane into the vacuole for sequestration of the metal (Kobae *et al.* 2004; Desbrosses-Fonrouge *et al.* 2005; Krämer 2005a). Additionally, a function for AtMTP1 in metal accumulation was suggested (Desbrosses-Fonrouge *et al.* 2005; Krämer 2005a). In addition, this protein was found to be highly Zn specific as no induction of *AtMTP1* transcription was observed upon exposure to excess Cd, Co, Cu, Fe or Mn (Desbrosses-Fonrouge *et al.* 2005).

The *T. caerulescens AtMTP1* ortholog *ZTP1* gene was identified from cDNA library (Assunçao *et al.* 2001). *ZTP1* expression levels were significantly higher in three *T. caerulescens* accessions, showing different accumulation and tolerance properties, than in the non-tolerant non-accumulator *T. arvense* upon exposure to different Zn concentrations (0, 2 and 10 µM Zn) and the most tolerant population also showed the highest expression (Assunçao *et al.* 2001). The authors concluded that high *ZTP1* expression might contribute to Zn tolerance in *T. caerulescens* (Assunçao *et al.* 2001). The *A. halleri AtMTP1* ortholog was intensively studied with regard to its implication in Zn homeostasis following its identification through the complementation of a Zn-hypersensitive yeast strain (Dräger *et al.* 2004). Consistent with *AtMTP1* localisation in the tonoplast (Kobae *et al.* 2004; Desbrosses-Fonrouge *et al.* 2005), *AhMTP1* localised exclusively in the vacuolar membrane of yeast cells and *A. thaliana* protoplasts (Dräger *et al.* 2004). In the shoots a substantially higher transcript level of *MTP1* was observed in *A. halleri* compared to the non-tolerant *A. thaliana* and *A. l. petraea* species, whereas in the roots no significant differences among the three species were observed in low Zn conditions at least (1 µM). Upon exposure for 4 days to high Zn concentrations (100 and 300 µM $ZnSO^4$ for the metallophyte and non-metallophyte species respectively) *MTP1* expression was upregulated in *A. halleri* while this was not observed in the two other species (Dräger *et al.* 2004). Similarly to *AtMTP1*, *AhMTP1* was highly specific to Zn (Dräger *et al.* 2004). Based on these findings *AhMTP1* was proposed to be involved in vacuolar sequestration of Zn in the roots and the shoots. The authors suggested that the low transcript levels of *AhMTP1* in the roots under low Zn conditions might indicate low rates of Zn vacuolar sequestration in the root cells under low Zn exposure, which should allow translocation of Zn to the shoots and thus, contribute to Zn hyperaccumulation (Dräger *et al.* 2004). At high Zn concentrations, enhanced *AhMTP1* expression in the roots was believed to contribute to the protection of the aboveground parts from accumulating excess Zn (Dräger *et al.* 2004). Circumstantial evidence for the implication of MTP1 in Zn tolerance in *A. halleri* was finally provided through the co-segregation of two of the three copies detected for AhMTP1 with Zn tolerance in an *A. halleri* x *A. l. petraea* backcross progeny (Dräger *et al.* 2004). The *AhMTP1* copies (MTP1-A and MTP1-B) that were apparently involved in Zn tolerance were expressed at much higher basal levels than the copy MTP1-C, which did not co-segregate with Zn

tolerance and were differentially regulated when compared to MTP1-C (Dräger *et al.* 2004).

TABLE 2.2 Metal transporter genes involved in metal homeostasis in *A. thaliana* and, potentially involved in metal tolerance and/or hyperaccumulation in *A. halleri* and *T. caerulescens*

Name	Metal specificity	Cellular localisation	Expression[a]	Predicted function[b]	Reference
			ZIP family		
A. thaliana					
IRT1	Fe (Cd, Co, Mn, Zn)	plasma membrane	in R under Fe deficiency	Fe uptake	Eide *et al.* 1996
ZIP1	Zn	plasma membrane	in R under Zn deficiency	Zn uptake	Grotz *et al.* 1998, Guerinot 2000
ZIP2	Zn, Cu, Cd	plasma membrane			Guerinot 2000
ZIP3	Zn	plasma membrane	in R under Zn deficiency	Zn uptake	Grotz *et al.* 1998
ZIP4	Zn	chloroplast membrane	in R and S under Zn deficiency	Zn transport between/within the cell	Grotz *et al.* 1998, Guerinot and Eide 1999
T. caerulescens					
ZNT1	Zn		higher in R and S compared to Ta	Zn uptake	Assunçao *et al.* 2001
A. halleri					
ZIP1	Zn		similar to At, R and S	(Zn uptake)	Becher *et al.* 2004

TABLE 2.2 (continued)

Name	Metal specificity	Cellular localisation	Expression[a]	Predicted function[b]	Reference
ZIP4	Zn		similar in S to At, higher in R than in At (high and low Zn)	(Zn transport)	Becher *et al.* 2004
ZIP6	Zn		higher in R and S than in At (low and high Zn)	(Zn transport)	Becher *et al.* 2004
ZIP9	Zn		higher in R than At under low Zn, downregulation under high Zn	(Zn transport)	Weber *et al.* 2004
		NRAMP family			
A. thaliana					
NRAMP1	Fe, Mn		upregulation in R under Fe deficiency	Fe transport	Curie *et al.* 2000
NRAMP3	Fe, Cd, Mn, Zn	vacuolar membrane	vascular tissue, R and S	mobilisation of metals from the vacuole	Thomine *et al.* 2000

TABLE 2.2 (continued)

Name	Metal specificity	Cellular localisation	Expression[a]	Predicted function[b]	Reference
NRAMP4	Fe, Mn		upregulation in R and S under Zn deficiency	Fe transport	Thomine *et al.* 2004
A. halleri					
NRAMP3			higher in R and S than in At under low Zn, no regulation by Zn treatment		Weber *et al.* 2004
			HMAs		
A. thaliana					
HMA2	Cd, Zn	plasma membrane	vascular tissue, R and S	xylem loading/unloading, mobilisation through phloem	Mills *et al.* 2003, Hussain *et al.* 2004,
HMA3	Cd	vacuolar membrane		sequestration	Gravot *et al.* 2004
HMA4	Cd, Zn	plasma membrane	vascular tissue, R and S	xylem loading/unloading, mobilisation through phloem	Verret *et al.* 2004, Verret *et al.* 2005

TABLE 2.2 (continued)

Name	Metal specificity	Cellular localisation	Expression[a]	Predicted function[b]	Reference
HMA5	Cu		predominant in R, induction by Cu in R and S	Cu detoxification in roots	Andrès-Colas *et al.* 2006
T. caerulescens					
HMA4	Cd, Zn		predominant in R	translocation of Cd and Zn	Bernard *et al.* 2004, Papoyan and Kochian 2004
A. halleri					
HMA3	Zn		predominant in S, higher in S (low Zn), similar in R (low Zn), upregulation under high Zn in R and S	(sequestration)	Becher *et al.* 2004
HMA4	Cd, Zn	plasma membrane	predominant in R, higher in R and S	protection of root cells	Courbot *et al.* submitted

TABLE 2.2 (continued)

Name	Metal specificity	Cellular localisation	Expression[a]	Predicted function[b]	Reference
			CDF family		
A. thaliana					
MTP1	Zn	vacuolar membrane	all tissues	vacuolar sequestration	Krämer 2005
T. caerulescens					
ZTP1	Zn		higher in S and R compared to At/Al	(vacuolar sequestration)	Assunçao *et al.* 2001
A. halleri					
MTP1	Zn	vacuolar membrane	predominant in S, upregulation in R and S under high Zn	vacuolar sequestration	Dräger *et al.* 2004

[a] Ta: *T. arvense;* At: *A. thaliana;* Al: *A. lyrata;* R: roots; S: shoots
[b] Predicted function of the gene, between brackets if function is based on the functional analysis in *A. thaliana* and the gene in *A. halleri* or *T. caerulescens* has not yet been characterised.

2.5 Conclusion

The genetic and physiological bases of heavy metal tolerance and hyperaccumulation in *A. halleri* have been addressed relatively recently. The first, genetic, analysis conducted on *A. halleri* has been reported by Macnair *et al.* in 1999 (Macnair *et al.* 1999) and this study constituted an important landmark in the current knowledge of heavy metal tolerance and hyperaccumulation in *A. halleri*. Macnair *et al.* (1999) investigated the genetic determinism of Zn tolerance and hyperaccumulation in *A. halleri* through the generation of a F2 progeny on a cross between *A. halleri* and its close non-tolerant non-accumulator relative *A. l. petraea*, which ensured the segregation of Zn tolerance and hyperaccumulation, otherwise constitutive traits in the *A. halleri* species. A single gene or few genes were suggested to govern Zn tolerance, whereas multiple genes were believed to be involved in Zn hyperaccumulation (Macnair *et al.* 1999). Additionally, the lack of co-segregation of Zn tolerance and hyperaccumulation in the F2 progeny indicated the existence of independent genetic mechanisms for both traits in the *A. halleri* species (Macnair *et al.* 1999). The genetic model of Cd tolerance in *A. halleri*, investigated in an *A. halleri* x *A. l. petraea* backcross progeny, was thought to be composed of two or three genes with additive effect (Bert *et al.* 2003). Furthermore, partially independent genetic mechanisms were suggested to be involved in Zn and Cd tolerance upon the analysis of the co-segregation of both traits in the backcross progeny (Bert *et al.* 2003). The study of the physiological mechanisms involved in metal tolerance and hyperaccumulation in *A. halleri* suggested a role in Zn tolerance and/or hyperaccumulation for the chelation of Zn by malate (Sarret *et al.* 2002). The metal homeostasis genes *AhZIP4*, *AhZIP6*, *AhZIP9*, *AhNRAMP3*, *AhHMA3*, *AhHMA4* and *AhMTP1* were reported to be higher expressed and/or differentially regulated in *A. halleri* when compared to other non-tolerant non-accumulator species, which was interpreted as a possible indication for their implication in metal tolerance and/or hyperaccumulation. However, with the exception of *AhMTP1* conclusive evidence for their role in Zn tolerance and/or hyperaccumulation in *A. halleri* could not be provided.

Although the studies described within this chapter revealed some interesting issues concerning the genetic and physiological bases of metal tolerance and hyperaccumulation in *A. halleri* many aspects remain elusive:

(a) How many genes are involved in metal tolerance in *A. halleri*?

(b) In addition to *AhMTP1* are there other genes involved in Zn tolerance and/or hyperaccumulation?

(c) Are the metal homeostasis genes, for which modifications were reported with regard to their expression and regulation in *A. halleri*, involved in metal tolerance and/or in hyperaccumulation?

It is difficult, if not impossible to achieve a clear answer on these questions through classical genetic or physiological analyses. A statistical method was established in the 1980s, i.e. the Quantitative Trait Loci (QTL) mapping method (Lander and Botstein 1989) that offered the possibility to identify the genetic architecture, and eventually the physiological bases of complex traits without any *a priori* knowledge of the trait under study (Tanksley 1993; Mackay 2001; Kearsey and Luo 2003). In the following chapter we present the principles of the QTL mapping method (see **3.2**). Finally, we describe how we applied this approach on Zn and Cd tolerance in *A. halleri* (see **3.3**).

2.6 References

Al-Shehbaz, I. A., and O'Kane, S. L., 2002 Taxonomy and Phylogeny of Arabidopsis (Brassicaceae), pp. 1-22 in *The Arabidopsis Book.* American Society of Plant Biologists.

Andrès-Colas, N., Sancenon, V., Rodriguez-Navarro, S., Mayo, S., Thiele, D. J., Ecker, J. R., *et al.*, 2006 The Arabidopsis heavy metal P-type ATPase HMA5 interacts with metallochaperones and functions in copper detoxification of roots. *Plant Journal* **45:** 225-236.

Antonovics, J., Bradshaw, A. D., and Turner, R. G., 1971 Heavy metal tolerance in plants. *Advances in Ecological Research* **7:** 1-85.

Assunçao, A. G. L., da Costa Martins, P., de Folter, S., Vooijs, R., Schat, H., and Aarts, M. G. M., 2001 Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens. Plant Cell and Environment* **24:** 217-226.

Assunçao, A. G. L., Schat, H., and Aarts, M. G. M., 2003a *Thlaspi caerulescens*, an attractive model species to study heavy metal hyperaccumulation in plants. *New Phytologist* **159:** 351-360.

Assunçao, A. G. L., Ten Bookum, W. M., Nelissen, H. J. M., Vooijs, R., Schat, H., and Ernst, W. H. O., 2003b Differential metal-specific tolerance and accumulation patterns among *Thlaspi caerulescens* populations originating from different soil types. *New Phytologist* **159:** 411-419.

Assunçao, A. G. L., Ten Bookum, W. M., Nelissen, H. J. M., Vooijs, R., Schat, H., and Ernst, W. H. O., 2003c A cosegregation analysis of zinc (Zn) accumulation and Zn tolerance in the Zn hyperaccumulator *Thlaspi caerulescens. New Phytologist* **159:** 383-390.

Axelsen, K. B., and Palmgren, M. G., 2001 Inventory of the superfamily of P-type ion pumps in Arabidopsis. *Plant Physiology* **126:** 696-706.

Baxter, I., Tchieu, J., Sussman, M. R., Boutry, M., Palmgren, M. G., Gribskov, M., *et al.*, 2003 Genomic comparison of P-type ATPase ion pumps in Arabidopsis and rice. *Plant Physiology* **132:** 618-628.

Becher, M., Talke, I. N., Krall, L., and Krämer, U., 2004 Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. *Plant Journal* **37:** 251-268.

Bernard, C., Roosens, N., Czernic, P., Lebrun, M., and Verbruggen, N., 2004 A novel CPx-ATPase from the cadmium hyperaccumulator *Thlaspi caerulescens*. *FEBS Letters* **569:** 140-148.

Bert, V., Macnair, M. R., de Laguerie, P., Saumitou-Laprade, P., and Petit, D., 2000 Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri* (Brassicaceae). *New Phytologist* **146:** 225-233.

Bert, V., Bonnin, I., Saumitou-Laprade, P., de Laguerie, P., and Petit, D., 2002 Do *Arabidopsis halleri* from nonmetallicolous populations accumulate zinc and cadmium more effectively than those from metallicolous populations? *New Phytologist* **155:** 47-57.

Bert, V., Meerts, P., Saumitou-Laprade, P., Salis, P., Gruber, W., and Verbruggen, N., 2003 Genetic basis of Cd tolerance and hyperaccumulation in *Arabidopsis halleri*. *Plant and Soil* **249:** 9-18.

Bleeker, P. M., 2004 Mechanisms of arsenic tolerance in higher plants, pp. 95 in *Faculteit der Aard- en Levenswetenschappen*. Vrije Universiteit Amsterdam, Amsterdam.

Bradshaw, A. D., 1970 Plants and industrial waste. *Transactions of the Botanical Society of Edinburgh* **41:** 71-84.

Brooks, R. R., 1998 *Plants that hyperaccumulate heavy metals*. Cab International, Wallingford.

Chardonnens, A. N., Koevoets, P. L. M., van Zanten, A., Schat, H., and Verkleij, J. A. C., 1999 Properties of enhanced tonoplast zinc transport in naturally selected zinc-tolerant *Silene vulgaris*. *Plant Physiology* **120:** 779-785.

Clemens, S., 2001 Molecular mechanisms of plant metal tolerance and homeostasis. *Planta* **212:** 475-486.

Clemens, S., Palmgren, M. G., and Krämer, U., 2002 A long way ahead: understanding and engineering plant metal accumulation. *Trends in Plant Science* **7:** 309-315.

Cobbett, C., and Goldsbrough, P., 2002 Phytochelatines and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annual Review of Plant Biology* **53:** 159-182.

Courbot, M., Willems, G., Motte, P., Arfvidsson, S., Saumitou-Laprade, P., and Verbruggen, N., submitted to *Plant Journal* The major QTL for cadmium tolerance in *Arabidopsis halleri* co-localizes with *HMA4*, a gene encoding a Heavy Metal ATPase.

Curie, C., Alonso, J. M., Le Jean, M., Ecker, J. R., and Briat, J.-F., 2000 Involvement of NRAMP1 from *Arabidopsis thaliana* in iron transport. *Biochemical Journal* **347:** 749-755.

de Knecht, J. A., van Baren, N., Ten Bookum, W. M., Wong Fong Sang, H. W., Koevoets, P. L. M., Schat, H., *et al.*, 1995 Synthesis and degradation of phytochelatins in cadmium-sensitive and cadmium-tolerant *Silene vulgaris*. *Plant Science* **106:** 9-18.

De Vos, C. H. R., Schat, H., De Waal, M. A. M., Vooijs, R., and Ernst, W. H. O., 1991 Increased resistance to copper-induced damage of the root cell plasmalemma in copper tolerant *Silene cucubalus*. *Physiologia Plantarum* **82:** 523-528.

Desbrosses-Fonrouge, A.-G., Voigt, K., Schröder, A., Arrivault, S., Thomine, S., and Krämer, U., 2005 *Arabidopsis thaliana* MTP1 is a Zn transporter in the vacuolar membrane which mediates Zn detoxification and drives leaf Zn accumulation. *FEBS Letters* **579:** 4165-4174.

Dräger, D. B., Desbrosses-Fonrouge, A.-G., Krach, C., Chardonnens, A. N., Meyer, R. C., Saumitou-Laprade, P., *et al.*, 2004 Two genes encoding *Arabidopsis halleri* MTP1 metal transport proteins co-segregate with zinc tolerance and account for high *MTP1* transcript levels. *Plant Journal* **39:** 425-439.

Eide, D., Broderius, M., Fett, J., and Guerinot, M. L., 1996 A novel iron-regulated metal transporter from plants identified by functional expression in yeast. *Proceedings of the National Academy of Sciences of the U S A* **93:** 5624-5628.

Feder, M. E., and Mitchell-Olds, T., 2003 Evolutionary and ecological functional genomics. *Nature Reviews Genetics* **4:** 649-655.

Frerot, H., Lefebvre, C., Petit, C., Collin, C., Dos Santos, A., and Escarre, J., 2005 Zinc tolerance and hyperaccumulation in F1 and F2 offspring from intra and interecotype crosses of *Thlaspi caerulescens*. *New Phytologist* **165:** 111-119.

Gravot, A., Lieutaud, A., Verret, F., Auroy, P., Vavasseur, A., and Richaud, P., 2004 AtHMA3, a plant P1B-ATPase, functions as a Cd/Pb transporter in yeast. *FEBS Letters* **561:** 22-28.

Gregory, R. P. G., and Bradshaw, A. D., 1964 Heavy metal tolerance in populations of *Agrostis tenuis* Sibth. and other grasses. *New Phytologist* **64:** 131-143.

Grotz, N., Fox, T., Connolly, E., Park, W., Guerinot, M. L., and Eide, D., 1998 Identification of a family of zinc transporter genes from Arabidopsis that respond to zinc deficiency. *Proceedings of the National Academy of Sciences of the U S A* **95:** 7220-7224.

Guerinot, M. L., and Eide, D., 1999 Zeroing in on zinc uptake in yeast and plants. *Current Opinion in Plant Biology* **2:** 244-249.

Guerinot, M. L., 2000 The ZIP family of metal transporters. *Biochimica et Biophysica Acta* **1465:** 190-198.

Hall, J. L., 2002 Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of Experimental Botany* **53:** 1-11.

Hall, J. L., and Williams, L. E., 2003 Transition metal transporters in plants. *Journal of Experimental Botany* **54:** 2601-2613.

Hanikenne, M., Kramer, U., Demoulin, V., and Baurain, D., 2005 A comparative inventory of metal transporters in the green alga Chlamydomonas reinhardtii and the red alga Cyanidioschizon merolae. *Plant Physiology* **137:** 428-446.

Harmens, H., Den Hartog, P. R., Bookum, W., and Verkleij, J., 1993 Increased Zinc Tolerance in *Silene vulgaris* (Moench) Garcke Is Not Due to Increased Production of Phytochelatins. *Plant Physiology* **103:** 1305-1309.

Hartley-Whitaker, J., Ainsworth, G., Vooijs, R., Ten Bookum, W., Schat, H., and Meharg, A. A., 2001 Phytochelatins are involved in differential arsenate tolerance in *Holcus lanatus*. *Plant Physiology* **126:** 299-306.

Hussain, D., Haydon, M. J., Wang, Y., Wong, E., Sherson, S. M., Young, J., *et al.*, 2004 P-Type ATPase Heavy Metal Transporters with roles in essential zinc homeostasis in Arabidopsis. *Plant Cell* **16:** 1327-1339.

Jowett, D., 1964 Population studies on lead tolerant *Agrostis tenuis*. *Evolution* **18:** 70-80.

Kearsey, M. J., and Luo, Z. W., 2003 Mapping, characterization and deployment of quantitative trait loci in *Plant Molecular Breeding*, edited by H. J. Newbury. CRC Press, Birmingham.

Kobae, Y., Uemura, T., Sato, M. H., Ohnishi, M., Mimura, T., Nakagawa, T., *et al.*, 2004 Zinc transporter of *Arabidopsis thaliana* AtMTP1 is localized to vacuolar membranes and implicated in zinc homeostasis. *Plant and Cell Physiology* **45:** 1749-1758.

Koch, M., Haubold, B., and Mitchell-Olds, T., 2001 Molecular systematics of the Brassicaceae: evidence from coding plastidic matK and nuclear Chs sequences. *American Journal of Botany* **88:** 534-544.

Krämer, U., Cotter-Howells, J. D., Charnock, J. M., Baker, A. J. M., and Smith, A. C., 1996 Free histidine as a metal chelator in plants that accumulate nickel. *Nature* **379:** 635-638.

Krämer, U., Smith, R. D., Wenzel, W. W., and Raskin, I., 1997 The rate of metal transport and tolerance in nickel hyperaccumulation by *Thlaspi goesingense* Halacsy. *Plant Physiology* **115:** 1641-1650.

Krämer, U., Pickering, I. J., Prince, R. C., Raskin, I., and Salt, D. E., 2000 Subcellular localization and speciation of nickel in hyperaccumulator and non-accumulator Thlaspi species. *Plant Physiology* **122:** 1343-1353.

Krämer, U., 2005a MTP1 mops up excess zinc in *Arabidopsis* cells. *Trends in Plant Science* **10:** 313-315.

Krämer, U., 2005b Phytoremediation: novel approaches to cleaning up polluted soils. *Current Opinion in Biotechnology* **16:** 133-141.

Kuittinen, H., de Haan, A. A., Vogl, C., Oikarinen, S., Leppala, J., Koch, M., *et al.*, 2004 Comparing the linkage maps of the close relatives *Arabidopsis lyrata* and *A. thaliana*. *Genetics* **168:** 1575-1584.

Küpper, H., Zhao, F. J., and McGrath, S. P., 1999 Cellular compartmentation of zinc in the leaves of the hyperaccumulator *Thlaspi caerulescens*. *Plant Physiology* **119:** 305-311.

Küpper, H., Lombi, E., Zhao, F. J., and McGrath, S. P., 2000 Cellular compartimentation of cadmium and zinc in relation to other elements in the hyperaccumulator *Arabidopsis halleri*. *Planta* **212:** 75-84.

Lander, E. S., and Botstein, D., 1989 Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. *Genetics* **121:** 185-199.

Lasat, M. M., Baker, A. J. M., and Kochian, L. V., 1996 Physiological characterization of root Zn^{2+} absorption and translocation to shoots in Zn hyperaccumulator and nonaccumulator species of *Thlaspi*. *Plant Physiology* **112:** 1715-1722.

Lasat, M. M., Baker, A. J. M., and Kochian, L. V., 1998 Altered Zn compartmentation in the root symplasm and stimulated Zn absorption into the leaf as mechanisms involved in Zn hyperaccumulation in *Thlaspi caerulescens*. *Plant Physiology* **118:** 875-883.

Lasat, M. M., Pence, N. S., Garvin, D. F., Ebbs, S. D., and Kochian, L. V., 2000 Molecular physiology of zinc transport in the Zn hyperaccumulator Thlaspi caerulescens. *Journal of Experimental Botany* **51:** 71-79.

Mackay, T. F., 2001 The genetic architecture of quantitative traits. *Annual Review of Genetics* **35:** 303-339.

Macnair, M. R., 1983 The genetic control for copper tolerance in the yellow monkey flower, Mimulus guttatus. *Heredity* **50:** 283-293.

Macnair, M. R., 1993 Tansley review No. 49: The genetics of metal tolerance in vascular plants. *New Phytologist* **124:** 541-559.

Macnair, M. R., Bert, V., Huitson, S. B., Saumitou-Laprade, P., and Petit, D., 1999 Zinc tolerance and hyperaccumulation are genetically independent characters. *Proceedings of the Royal Society of London, Series B: Biological Sciences* **266:** 2175-2179.

Macnair, M. R., Tilstone, G. H., and Smith, S. E., 2000 The genetics of metal tolerance and accumulation in higher plants, pp. 235-250 in *Phytoremediation of contaminated soil and water*, edited by C. Press.

Mäser, P., Thomine, S., Schroeder, J. I., Ward, J. M., Hirschi, K., Sze, H., *et al.*, 2001 Phylogenetic relationships within cation transporter families of Arabidopsis. *Plant Physiology* **126:** 1646-1667.

Meharg, A. A., and Macnair, M. R., 1992 Suppression of the high-affinity phosphate-uptake system: a mechanism of arsenate tolerance in *Holcus lanatus* L. *Journal of Experimental Biology* **43:** 519-524.

Meharg, A. A., 1994 Integrated tolerance mechanisms - constitutive and adaptive plant - responses to elevated metal concentrations in the environment. *Plant, Cell and Environment* **17:** 989-993.

Mills, R., Krijger, G., Baccarini, P., Hall, J. L., and Williams, L., 2003 Functional expression of AtHMA4, a P_{1B}-type ATPase of the Zn/Co/Cd/Pb subclass. *Plant Journal* **35:** 164-176.

Mitchell-Olds, T., 2001 *Arabidopsis thaliana* and its wild relatives: a model system for ecology and evolution. *Trends in Ecology and Evolution* **16:** 693-700.

Murphy, A., and Taiz, L., 1995 Comparison of Metallothionein gene expression and nonprotein thiols in ten Arabidopsis ecotypes. *Plant Physiology* **109:** 945-954.

Papoyan, A., and Kochian, L. V., 2004 Identification of *Thlaspi caerulescens* genes that may be involved in heavy metal hyperaccumulation and tolerance. Characterization of a novel heavy metal transporting ATPase. *Plant Physiology* **136:** 3814-3823.

Pauwels, M., Saumitou-Laprade, P., Holl, A., Petit, D., and Bonnin, I., 2005 Multiple origin of metallicolous populations of the pseudometallophyte *Arabidopsis halleri* (Brassicaceae) in central Europe: the cpDNA testimony. *Molecular Ecology* **14:** 4403-4414.

Pence, N. S., Larsen, P. B., Ebbs, S. D., Letham, D. L., Lasat, M. M., Garvin, D. F., *et al.*, 2000 The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. *Proceedings of the National Academy of Sciences of the U S A* **97:** 4956-4960.

Salt, D. E., Smith, R. D., and Raskin, I., 1998 Phytoremediation. *Annual Review of Plant Physiology and Plant Molecular Biology* **49:** 643-668.

Sarret, G., Saumitou-Laprade, P., Bert, V., Proux, O., Hazemann, J. L., Traverse, A., *et al.*, 2002 Forms of zinc accumulated in the hyperaccumulator *Arabidopsis halleri*. *Plant Physiology* **130:** 1815-1826.

Schat, H., and Ten Bookum, W. M., 1992 Genetic control of copper tolerance in *Silene vulgaris*. *Heredity* **68:** 219-229.

Schat, H., Kuiper, E., Ten Bookum, W. M., and Vooijs, R., 1993 A general model for the genetic control of copper tolerance in *Silene vulgaris*: evidence from crosses between plants from different tolerant populations. *Heredity* **70:** 142-147.

Schat, H., Vooijs, R., and Kuiper, E., 1996 Identical major gene loci for heavy metal tolerances that have independently evolved in different local populations and subspecies of *Silene vulgaris*. *Evolution* **50:** 1888-1895.

Schat, H., and Vooijs, R., 1997 Multiple tolerance and co-tolerance to heavy metals in *Silene vulgaris*: a co-segregation analysis. *New Phytologist* **136:** 489-496.

Schat, H., Llugany, M., Vooijs, R., Hartley-Whitaker, J., and Bleeker, P. M., 2002 The role of phytochelatins in constitutive and adaptive heavy metal tolerances in hyperaccumulator and non-hyperaccumulator metallophytes. *Journal of Experimental Botany* **53:** 2381-2392.

Tanksley, S. D., 1993 Mapping polygenes. *Annual Review of Genetics* **27:** 205-233.

Thomine, S., Wang, R., Ward, J. M., Crawford, N. M., and Schroeder, J. I., 2000 Cadmium and iron transport by members of a plant metal transporter family in Arabidopsis with homology to Nramp genes. *Proceedings of the National Academy of Sciences of the U S A* **97:** 4991-4996.

Tilstone, G. H., and Macnair, M. R., 1997 Nickel tolerance and copper - nickel co-tolerance in Mimulus guttatus from copper mine and serpentine habitats. *Plant and Soil* **191:** 173-181.

van der Zaal, B. J., Neuteboom, L. W., Pinas, J. E., Chardonnens, A. N., Schat, H., Verkleij, J. A. C., *et al.*, 1999 Overexpression of a novel Arabidopsis gene related to putative zinc-transporter genes from animals can lead to enhanced zinc resistance and accumulation. *Plant Physiology* **119:** 1047-1055.

van Hoof, N. A. L. M., Hassinen, V. H., Hakvoort, H. W. J., Ballintijn, K. F., Schat, H., Verkleij, J. A. C., *et al.*, 2001a Enhanced copper tolerance in *Silene vulgaris* (Moench) Garcke populations from copper mines is associated with increased transcript levels of a 2b-type metallothionein gene. *Plant Physiology* **126:** 1519-1526.

van Hoof, N. A. L. M., Koevoets, P. L. M., Hakvoort, H. W. J., Ten Bookum, W. M., Schat, H., Verkleij, J. A. C., *et al.*, 2001b Enhanced ATP-dependent copper efflux across the root cell plasma membrane in copper-tolerant *Silene vulgaris*. *Physiologia Plantarum* **113:** 225-232.

Van Rossum, F., Bonnin, I., Fenart, S., Pauwels, M., Petit, D., and Saumitou-Laprade, P., 2004 Spatial genetic structure within a metallicolous population of

Arabidopsis halleri, a clonal, self-incompatible and heavy-metal-tolerant species. *Molecular Ecology* **13:** 2959-2967.

Verkleij, J. A. C., Koevoets, P. L. M., Blake-Kalff, M. M. A., and Chardonnens, A. N., 1998 Evidence for an important role of the tonoplast in the mechanism of naturally selected Zn tolerance in *Silene vulgaris*. *Journal of Plant Physiology* **153**.

Verret, F., Gravot, A., Auroy, P., Leonhardt, N., David, P., Nussaume, L., *et al.*, 2004 Overexpression of AtHMA4 enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. *FEBS Letters* **576:** 306-312.

Verret, F., Gravot, A., Auroy, P., Preveral, S., Forestier, C., Vavasseur, A., *et al.*, 2005 Heavy metal transport by AtHMA4 involves the N-terminal degenerated metal binding domain and the C-terminal His11 stretch. *FEBS Letters* **579:** 1515-1522.

Watkins, A. J., and Macnair, M. R., 1991 Genetics of arsenic tolerance in *Agrostis capillaris* L. *Heredity* **66:** 47-54.

Weber, M., Harada, E., Vess, C., Roepenack-Lahaye, E., and Clemens, S., 2004 Comparative microarray analysis of *Arabidopsis thaliana* and *Arabidopsis halleri* roots identifies nicotianamine synthase, a ZIP transporter and other genes as potential metal hyperaccumulation factors. *Plant Journal* **37:** 269-281.

Wilkins, D. A., 1978 The measurement of tolerance to edaphic factors by means of root growth. *New Phytologist* **80:** 623-634.

Willems, G., Godé, C., Dräger, D., Courbot, M., Verbruggen, N., and Saumitou-Laprade, P., submitted to *Genetics* Quantitative Trait Loci mapping of zinc tolerance in the metallophyte *Arabidopsis halleri* ssp. *halleri*.

Yogeeswaran, K., Frary, A., York, T. L., Amenta, A., Lesser, A. H., Nasrallah, J. B., *et al.*, 2005 Comparative genome analyses of *Arabidopsis* spp.: Inferring chromosomal rearrangement events in the evolutionary history of *A. thaliana*. *Genome Research* **15:** 505-515.

Zhou, J., and Goldsbrough, P. B., 1995 Structure, organization and expression of the metallothionein gene family in Arabidopsis. *Molecular Genetics and Genomics* **248:** 318-328.

3 THE QUANTITATIVE TRAIT LOCI MAPPING APPROACH

3.1 Introduction to Quantitative Trait Loci mapping

According to Mendel's experiments, the genotype at a locus could be unambiguously inferred from the phenotype of an individual. Loci underlying traits for which the wild phenotype could be easily distinguished from the mutant phenotype showed to carry macromutations, i.e. mutations with large phenotypic effects. In natural populations however, macromutations are expected to be rare because they should be readily outcompeted given their large phenotypic effect. Indeed, most traits in nature show continuous instead of discrete variation. These traits are assumed to be under the control of several, sometimes many loci, each with a relatively small phenotypic effect, interacting with each other and with the environment (Fisher 1918). The genes underlying traits showing continuous variation were referred to as polygenes. Since the 1980s they were currently named quantitative trait loci (QTL).

In 1923, Sax reported the association of seed size in beans (a continuous trait) with seed-coat pigmentation (a monogenic discrete trait) (Sax 1923). He introduced the basic principle of QTL detection, i.e. the identification of linkage between the gene underlying a monogenic trait and one or more of the loci underlying a continuous trait. However, the number of monogenic markers showing a visible effect on the phenotype was limited for most organisms and most of the genes underlying monogenic traits interfered with the continuous trait of interest. In the 1980s, a new type of marker, iso- or allozymes, was discovered based on the separation of the different allelic forms of enzymes upon their electrophoretic mobility. The number of markers available for an organism was highly increased because they did not need anymore to cause a discrete and visible change in the phenotype of the organism. Simultaneously, the risk of

interference between the marker and the continuous trait was highly reduced. Through the survey of genetic variation at the DNA level, much more polymorphisms could be observed than for enzymes and an even greater number of markers was now available. The first DNA-markers were revealed through Restriction Fragment Length Polymorphism (RFLP). With the development of the Polymerase Chain Reaction (PCR) other DNA-markers, such as mini- and microsatellites, Amplified Fragment Length Polymorphisms (AFLPs) and Single Nucleotide Polymorphisms (SNPs) have been introduced (Table 3.1). The significant increase of markers made the construction of genetic linkage maps covering entire genomes possible. In order to exploit these data efficiently the development of reliable statistical methodologies for QTL detection was highly stimulated. The construction of detailed genetic maps provided the foundation for the modern-day QTL mapping methodologies.

TABLE 3.1: DNA markers: variation is either revealed through nucleic acid hybridisation or amplification

	Nucleic acid hybridisation		Amplification	
Marker	**Restriction Fragment Length Polymorphism**	**Mini- and microsatellites[a]**	**Amplified Fragment Length Polymorphism**	**Single Nucleotide Polymorphism**
Polymorphism	Restriction site variation producing fragment length polymorphism	Length polymorphism	Restriction site variation producing presence/absence of PCR fragment	Single point mutations
Revelation method	1.Digestion of DNA and separation of DNA fragments by electrophoresis on agarose gels 2.Denaturation of DNA fragments and blotting on membrane 3.Hybridization of DNA fragments with specific probes	Amplification	1.Digestion of DNA (rare and frequent cutter) and ligation 2.Pre-amplification: 3.Selective amplification (will be detailed in **4.2**)	Sequencing Mass spectrometry
Characteristics	* Codominant * Sequence of probe generally known * Study of genome homology between different species possible	* Codominant * Highly polymorphic through variation in repeat number * Sequence knowledge of specific flanking regions required * Study of genome homology between different species possible	* Dominant * Occasionally codominant through differences in band intensities * Sequence knowledge not required	* Codominant * Sequence knowledge required

Four different types of polymorphic markers are represented. Other DNA markers are for instance Cleaved Amplified Polymorphic Sequences (CAPS), Random Amplified Polymorphic DNA markers (RAPD) or Single-Strand Conformational Polymorphisms (SSCPs).
[a] tandem repeats of 9-100 bp and 1-6 bp for mini- and microsatellites respectively

3.2 Principle of Quantitative Trait Loci mapping

The estimation of the number and genome position of segregating QTLs in a mapping population is referred to as QTL mapping (Tanksley 1993). The principle underlying QTL mapping is based on the existence of linkage disequilibrium between alleles at a marker locus and alleles at a QTL. Linkage disequilibrium, defined as the non-random association of alleles at different loci, is a common phenomenon in nature (Tanksley 1993; Mackay 2001). In natural conditions linkage disequilibrium is caused by factors such as random drift, selection or migration. In the context of QTL mapping, linkage disequilibrium is generated by physical linkage between loci in populations derived from crosses. In summary, the mapping of QTLs underlying a quantitative trait requires (a) the generation of a progeny segregating for the trait of interest, i.e. the mapping population, (b) its characterisation for molecular markers, i.e. the construction of the genetic linkage map and, (c) the search for linkage disequilibrium between the marker loci and the quantitative trait through the combination of phenotyping and genotyping data (Figure 3.1) (Tanksley 1993; Mackay 2001; Kearsey and Luo 2003).

In so-called "primary or coarse QTL mapping experiments", QTLs are localised on the genetic linkage map within an interval of 5 to 30 centiMorgan (cM) (Glazier *et al.* 2002; Abiola *et al.* 2003; Salvi and Tuberosa 2005). A more accurate estimation of the QTL position can be achieved through fine mapping (Yano 2001; Glazier *et al.* 2002; Abiola *et al.* 2003; Kearsey and Luo 2003). Finally, the gene(s) underlying the QTL can be identified and ideally the polymorphism within the gene(s), i.e. the Quantitative Trait Nucleotide (QTN) causing the difference in the trait phenotype (Flint and Mott 2001; Mackay 2001; Salvi and Tuberosa 2005).

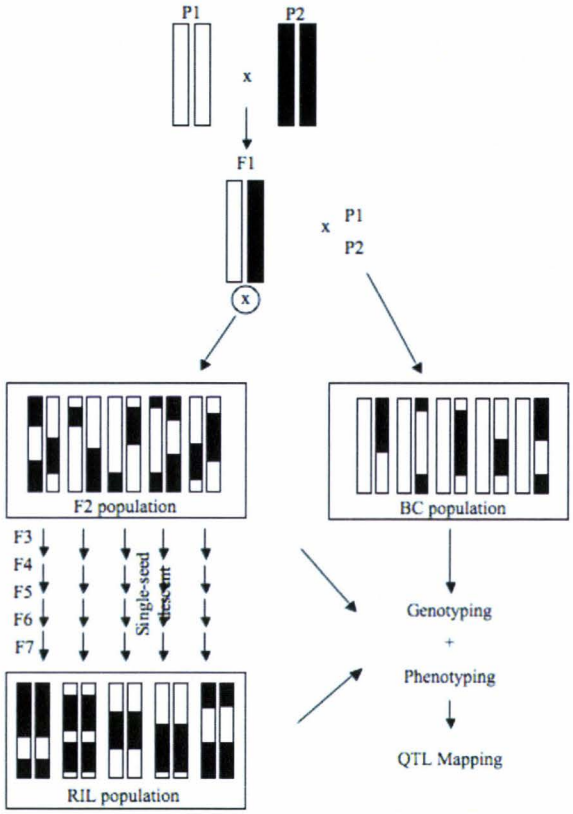


FIGURE 3.1 Different steps performed in a QTL mapping experiment, according to Alonso-Blanco and Koornneef 2000.

The construction of a mapping population (F2, backcross (BC) or Recombinant Inbred Line (RIL)), genotyping of the mapping population with molecular markers, phenotyping of the mapping population for the trait of interest, and finally the combination of both data sets to search for significant linkage disequilibrium between molecular markers and the quantitative trait, i.e. QTL mapping.
P1 and P2: parental lines used for the development of the mapping population;
F1: hybrid obtained by crossing the parental lines;
BC: backcross population, obtained by crossing an F1 individual to one of the parental lines (here crossing to P2);
F2: F2 population, obtained through selfing an F1 individual;
RIL: Recombinant Inbred Line obtained by single-seed descent from an F2 population followed by several generations (F3-F7) of selfing.

3.2.1 Mapping populations

The development of a mapping population constitutes the first step in QTL mapping. Inbred line crosses are most powerful in QTL mapping studies because in these crosses the linkage disequilibrium between the markers and the quantitative trait is

maximized (Tanksley 1993; Mackay 2001; Kearsey and Luo 2003). Ideally, two individuals are employed that are highly differentiated for both the trait of interest and the molecular markers to generate a large segregation of the trait in the mapping population. The lack of phenotypic variation between the parental individuals however, does not preclude the existence of genetic variation and can also result in large segregation of the trait in the mapping population (Liu 1998; Mauricio 2001).

Mostly used in QTL mapping experiments are backcross and F2 progenies (Figure 3.1) (Tanksley 1993; Kearsey and Luo 2003). The major advantage of the F2 design is that three genotypes are present at every marker in the mapping population and can be distinguished if codominant markers are used. On the contrary, only two genotypes are possible at each locus in a backcross population. In F2 populations dominance and additive effects can be estimated at the QTL position whereas backcross populations only provide an estimation of the additive component at the QTL position (Tanksley 1993; Mackay 2001; Kearsey and Luo 2003). Since the past decade the use of Recombinant Inbred Lines (RILs) in QTL mapping studies has highly increased (Alonso-Blanco and Koornneef 2000; Loudet *et al.* 2002). RILs are so-called "immortal" populations, produced by selfing of F2 individuals (single-seed descent method) for 5 to 8 generations (Figure 3.1); these lines are all homozygous and thus, fixed throughout the entire genome. Phenotyping data can be generated for a large number of traits or for the same trait but in different environments, while the genotyping of the mapping population has to be performed only once. Additionally, phenotyping data for one trait in one environment can be obtained on multiple replicates, reducing the environmental effects and increasing the power of QTL detection. To date, the employment of RILs has been predominantly restricted to the current model species *A. thaliana* (van Der Schaar *et al.* 1997; Alonso-Blanco *et al.* 1998; Loudet *et al.* 2003; Loudet *et al.* 2005, Gardner and Latta 2006).

3.2.2 Linkage map construction

The construction of a genetic linkage map involves

(a) the estimation of recombination frequencies between all pairs of loci, with which the mapping population has been genotyped, by the method of maximum likelihood (Liu 1998);

(b) the separation of loci in linkage groups or groups of loci which are inherited together on the basis of a logarithm of odds (LOD) score. The LOD score corresponds to the 10-based logarithm of the ratio of, the probability of linkage between two loci at the recombination value for which the likelihood reaches its maximum (the alternative hypothesis) over the probability that the two loci are not linked (the null hypothesis) (Stam 1993). A LOD score of 3 for instance means that the chances are greater than 1000:1 that the loci are linked for a given recombination estimate (Stam 1993). Grouping of loci can be done at several critical LOD values; a critical LOD value of 3 is generally assumed to prevent incorrect assignment of markers to the same linkage groups (Stam 1993).

$$\text{LOD score} = \text{Log}\frac{L(r_A)}{L(r=0.5)}$$

in which $L(r_A)$: the likelihood of the observations taking a value $r = r_A$ which maximizes the likelihood and $L(r = 0.5)$: the likelihood of the observations at a value $r = 0.5$, i.e. under the absence of linkage (the null hypothesis);

(c) the ordering of the loci on the linkage group. Several computational methods have been developed in this aim given the large number of possible gene orders[2] within a linkage group (Liu 1998);

(d) the transformation of recombination frequencies into genetic distances, expressed in (centi)Morgan (cM) (Liu 1998). The Haldane's and Kosambi's mapping function are currently implemented in the software programs for linkage map construction. The Haldane's mapping function assumes that crossovers occur randomly along the length of the chromosome. In contrast to the Haldane's mapping function, the Kosambi's mapping function includes genetic interference, which consists in the

[2] For L loci, the number of possible orders equals L!/2.

assumption that for three loci in the order ABC crossovers between the loci A and B are not independent of those occurring between the loci B and C (Liu 1998).

3.2.3 Quantitative Trait Loci mapping methods

3.2.3.1 Single marker analysis

The detection of QTLs in single marker analysis consists in comparing the means for the phenotypic trait among the genotypic classes at one marker locus (Tanksley 1993; Liu 1998) (see Annexes **BOX 3**). A significant difference between the phenotypic means revealed through a comparison-of-means test (t-test, analysis of variance, non-parametric test, etc) indicates then the presence of linkage between the marker and the QTL (Tanksley 1993; Liu 1998). Single marker analysis has the advantage over the other QTL mapping methods that it does not require the construction of a genetic linkage map for the mapping population. However, a number of shortcomings characterise this method (Lander and Botstein 1989; Tanksley 1993; Kearsey and Farquhar 1998):

(a) Because of linkage between the marker loci, the statistical tests performed on each of the markers are not independent. Consequently, while at a threshold value α of 0.05 the false positive rate at any given marker is 5%, the chance that at least one false positive will occur somewhere in the genome is much higher. In order to reduce the number of false positives a more stringent threshold value has to be adopted[3].

(b) The phenotypic effect of any detected QTL may be seriously underestimated through misclassification of individuals because of recombination between the marker locus and the QTL. Consequently, the further a QTL is located from the marker locus, the less probable it is to detect the QTL statistically. These problems are minimized through an increase of the number of segregating loci, since any potential QTL would then be closely linked to at least one marker locus.

[3] In order to obtain a genome-wide significance level of 0.05, the significance level adopted at a single marker can be moved downwards by applying for instance the Bonferroni correction for multiple tests. According to the Bonferroni correction, the significance level at a marker is obtained by dividing the genome-wide significance level of 0.05 by the number of tests being performed (Lander and Botstein 1989; Kearsey and Farquhar 1998).

I Single marker analysis does not provide an indication on the position of the QTL in the genome. Moreover, this method cannot distinguish between a QTL with small effect tightly linked to the marker locus or a QTL with large effect but weakly linked to the marker locus.

3.2.3.2 Interval mapping

The interval mapping (IM) method uses the information provided by two markers through the scanning of intervals between adjacent pairs of markers along a chromosome (or linkage group) (Lander and Botstein 1989) (see Annexes **BOX 3**). At each position within the interval, for instance at each cM, the probability of the presence of a QTL is obtained through the calculation of a LOD score, which evaluates the maximum likelihood of the observations under the assumption that a QTL is present (the alternative hypothesis) and compares it to the maximum likelihood of the observations assuming the absence of a QTL at the tested position (the null hypothesis) (Lander and Botstein 1989).

$$\text{LOD score} = \text{Log}\frac{L_1}{L_0}$$

in which L_1 : the maximum likelihood of the observations under the presence of a QTL and L_0 : the maximum likelihood of the observations under the absence of a QTL.

The position at which the LOD score reaches its maximum and, exceeds the significance threshold corresponds to the most likely position of the QTL (BOX 3.1). Initially, Lander and Botstein (1989) proposed a LOD value of 2 to 3 to ensure a 5% overall false-positive rate. Currently, permutation tests (Churchill and Doerge 1994; Doerge and Churchill 1996) are assumed to provide better estimates of the LOD thresholds that have to be applied in the IM method (Doerge 2002; Abiola *et al.* 2003; Kearsey and Luo 2003; Erickson *et al.* 2004). In a permutation test, genome scans are repeatedly carried out on permutated or shuffled versions of the phenotypic data set while retaining all genotypic and population information (such as segregation distortion, mapping data and recombination fractions) to estimate a LOD threshold value that is the most appropriate for the given data set. Permutation analyses can thus provide threshold values even for non-standard phenotypic data sets, for instance those showing a non-

normal distribution (Doerge 2002; Kearsey and Luo 2003). Confidence intervals reflecting the approximate position of the QTL are given by one-LOD and two-LOD support (~95% confidence interval) intervals, corresponding respectively to a 10-fold and 100-fold decrease in the likelihood ratio (Lander and Botstein 1989; Kearsey and Luo 2003).

Through the use of linked markers, IM is able to compensate for recombination between the markers and the QTL leading to an increase in QTL detection power and a more precise estimate of the effect of the QTL (Lander and Botstein 1989; Tanksley 1993). Consequently, the maximum benefit of IM to single marker analysis is realised when linked markers are fairly far apart (> 20 cM) since in these conditions many recombination events between the marker and the QTL are likely to occur. For higher marker densities both methods give identical results (Lander and Botstein 1989; Tanksley 1993). Similarly to single marker analysis, interval mapping is a single QTL method; these methods imply only a one-dimensional search for QTLs due to the fact that they search for QTLs through the ordered genetic markers in a systematic, linear fashion. These methods do not allow the integration of the effects of other QTLs, which might reduce the power and precision of QTL mapping. In IM for instance, false identification of QTLs (ghost QTLs) can arise if other QTLs are closely linked to the interval of interest (Martinez and Curnow 1992).

3.2.3.3 Multiple Quantitative Trait Loci

Ideally, the search for QTLs should be a multi-dimensional search, i.e. a search for QTLs considering every position in the genome simultaneously. Additionally, the eventual interactions between the QTLs or between the QTLs and the environment should be taken into account. However to date, the application of such a concept is highly computationally demanding and not feasible. Methods referred to as composite interval mapping (CIM) (Zeng 1993; Zeng 1994) or multiple QTL mapping (MQM) (Jansen 1993; Jansen 1994; Jansen and Stam 1994) were established in which the use of other markers in addition to the flanking markers was proposed as a background control in IM analysis (Jansen 1993; Zeng 1993; Jansen 1994; Jansen and Stam 1994; Zeng 1994; Doerge 2002; Kearsey and Luo 2003) (see Annexes **BOX 3**). These markers or

cofactors are supposed to take over the role of the nearby QTLs. Consequently they reduce the residual variance through the removal of the variation that is explained by the other QTLs while testing for a single segregating QTL as in IM. Compared to the IM method, the application of CIM or MQM results in an increase in the power of QTL detection as well as in mapping precision (Jansen 1993; Zeng 1993; Jansen 1994; Jansen and Stam 1994; Zeng 1994; Doerge 2002)

3.3 Conclusion

Since most adaptive traits in nature show a continuous rather than discrete distribution, the QTL mapping approach (Lander and Botstein 1989) constitutes an important tool for examining the genetic architecture of these traits (Mauricio 2001; Olsen and Purugganan 2002; Orr 2005). Currently, QTL mapping is considered an efficient method in molecular biology for identifying the molecular basis of complex traits, as well as in evolutionary biology for providing information about the trait's evolutionary history in natural populations or species (Mitchell-Olds 1995; Mauricio 2001).

Zn and Cd tolerance are complex adaptive traits for which the genetic as well as physiological bases are largely unknown as previously demonstrated in this work. Making use of the proximity of the biological model species *A. thaliana*, as well as of the increasing knowledge on the genome structure of the *A. lyrata* subspecies, we applied a QTL mapping approach to investigate the genetic architecture of Zn tolerance (see chapter **4**) and Cd tolerance (see chapter **5**) in *A. halleri*. Moreover, through the mapping of metal homeostasis genes described in the literature to be potentially involved in adaptive metal tolerance, the QTL mapping method enabled us to analyse the implication of these genes in Zn and Cd tolerance in *A. halleri*. In addition, the construction of a genetic linkage map, on which we positioned predominantly sequence-based markers anchored in the *A. thaliana* genome provided us the opportunity to integrate results obtained through high-throughput "–omic" approaches and QTL analyses. Through the combination of transcriptomic and QTL analyses conducted on

Zn tolerance we identified positional candidate genes conferring Zn tolerance in *A. halleri* (see chapter 6).

3.4 References

Abiola, O., Angel, J. M., Avner, P., Bachmanov, A., Belknap, J. K., and Bennett, B., 2003 The nature and identification of quantitative trait loci: a community's view. *Nature Reviews Genetics* **4:** 911-916.

Alonso-Blanco, C., Peeters, A. J., Koornneef, M., Lister, C., Dean, C., van den Bosch, N., *et al.*, 1998 Development of an AFLP based linkage map of Ler, Col and Cvi Arabidopsis thaliana ecotypes and construction of a Ler/Cvi recombinant inbred line population. *Plant Journal* **14:** 259-271.

Alonso-Blanco, C., and Koornneef, M., 2000 Naturally occurring variation in *Arabidopsis*: an underexploited resource for plant genetics. *Trends in Plant Science* **5:** 22-29.

Churchill, G. A., and Doerge, R. W., 1994 Emperical threshold values for quantitative trait mapping. *Genetics* **138:** 963-971.

Doerge, R. W., and Churchill, G. A., 1996 Permutation tests for multiple loci affecting a quantitative character. *Genetics* **142:** 285-294.

Doerge, R. W., 2002 Mapping and analysis of quantitative trait loci in experimental populations. *Nature Reviews Genetics* **3:** 43-52.

Erickson, D. L., Fenster, C. B., Stenoien, H. K., and Price, D., 2004 Quantitative trait locus analyses and the study of evolutionary process. *Molecular Ecology* **13:** 2505-2522.

Fisher, R. A., 1918 The correlation between relatives on the suppression of Mendelian inheritance. *Transactions of the Royal Society of Edinburgh* **52:** 399-433.

Flint, J., and Mott, R., 2001 Finding the molecular basis of quantitive traits: successes and pitfalls. *Nature Reviews Genetics* **2:** 437-445.

Gardner, K. M. and Latta, R. G., 2006 Identifying loci under selection across contrasting environments in *Avena barbata* using quantitative trait locus mapping. *Molecular Ecology* **15:** 1321-1333.

Glazier, A. M., Nadeau, J. H., and Aitman, T. J., 2002 Finding genes that underlie complex traits. *Science* **298:** 2345-2349.

Jansen, R. C., 1993 Interval mapping of multiple quantitative trait loci. *Genetics* **135:** 205-211.

Jansen, R. C., 1994 Controlling the type I and type II errors in mapping quantitative trait loci. *Genetics* **138:** 871-881.

Jansen, R. C., and Stam, P., 1994 High resolution of quantitative traits into multiple loci via interval mapping. *Genetics* **136:** 1447-1455.

Kearsey, M. J., and Farquhar, A. G., 1998 QTL analysis in plants; where are we now? *Heredity* **80:** 137-142.

Kearsey, M. J., and Luo, Z. W., 2003 Mapping, characterization and deployment of quantitative trait loci in *Plant Molecular Breeding*, edited by H. J. Newbury. CRC Press, Birmingham.

Lander, E. S., and Botstein, D., 1989 Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. *Genetics* **121:** 185-199.

Liu, B. H., 1998 *Statistical genomics: Linkage, mapping and QTL analysis*. CRC Press, Florida.

Loudet, O., Chaillou, S., Camilleri, C., Bouchez, D., and Daniel-Vedele, F., 2002 Bay-0 Shahdara recombinant inbred line population: a powerful tool for the genetic dissection of complex traits in Arabidopsis. *Theoretical and Applied Genetics* **104:** 1173-1184.

Loudet, O., Chaillou, S., Merigout, P., Talbotec, J., and Daniel-Vedele, F., 2003 Quantitative trait Loci analysis of nitrogen use efficiency in Arabidopsis. *Plant Physiology* **131:** 345-358.

Loudet, O., Gaudon, V., Trubuil, A., and Daniel-Vedele, F., 2005 Quantitative trait loci controlling root growth and architecture in Arabidopsis thaliana confirmed by heterogeneous inbred family. *Theoretical and Applied Genetics* **110:** 742-753.

Mackay, T. F., 2001 The genetic architecture of quantitative traits. *Annual Review of Genetics* **35:** 303-339.

Martinez, O., and Curnow, R. N., 1992 Estimating the locations and the sizes of the effects of quantitative trait loci using flanking markers. *Theoretical and Applied Genetics* **85:** 480-488.

Mauricio, R., 2001 Mapping Quantitative Trait Loci in plants: uses and caveats for evolutionary biology. *Nature Reviews Genetics* **2:** 370-380.

Mitchell-Olds, T., 1995 The molecular basis of quantitative genetic variation in natural populations. *Trends in Ecology and Evolution* **10:** 324-328.

Olsen, K. M., and Purugganan, M. D., 2002 Plant population genomics, linkage disequilibrium mapping, and the genetics of adaptation *Plant Adaptation: Molecular Genetics and Ecology.*, edited by J. W. Q.C.B. Cronk, R. H. Ree and I.E.P. Taylor, Vancouver, British Columbia, Canada.

Orr, H. A., 2005 The genetic theory of adaptation: a brief history. *Nature Reviews Genetics* **6:** 119-127.

Salvi, S., and Tuberosa, R., 2005 To clone or not to clone plant QTLs: present and future challenges. *Trends in Plant Science* **10:** 297-304.

Sax, K., 1923 The association of size differences with seed-coat pattern and pigmentation in *Phaseolus vulgaris*. *Genetics* **8:** 552-560.

Stam, P., 1993 Construction of integrated genetic linkage maps by means of a new computer package: JOINMAP. *Plant Journal* **3:** 739-744.

Tanksley, S. D., 1993 Mapping polygenes. *Annual Review of Genetics* **27:** 205-233.

Van Der Schaar, W., Alonso-Blanco, C., Leon-Kloosterziel, K. M., Jansen, R. C., van Ooijen, J. W., and Koornneef, M., 1997 QTL analysis of seed dormancy in Arabidopsis using recombinant inbred lines and MQM mapping. *Heredity* **79:** 190-200.

Yano, M., 2001 Genetic and molecular dissection of naturally occurring variation. *Current Opinion in Plant Biology* **4:** 130-135.

Zeng, Z.-B., 1993 Theoretical basis of precision mapping of quantitative trait loci. *Proceedings of the National Academy of Sciences of the U S A* **90**.

Zeng, Z. B., 1994 Precision mapping of quantitative trait loci. *Genetics* **136:** 1457-1468.

Results

4 QUANTITATIVE TRAIT LOCI MAPPING OF ZINC TOLERANCE IN THE METALLOPHYTE *ARABIDOPSIS HALLERI*

Willems, G., Godé, C., Dräger, D. B., Courbot, M., Verbruggen, N., Saumitou-Laprade, P.

Submitted to Genetics

4.1 Introduction

The study of adaptive traits is a major challenge in evolutionary biology (Olsen and Purugganan 2002). Most studies have focused on model species since for these species a wide range of molecular resources are available (Alonso-Blanco and Koornneef 2000; Koornneef *et al.* 2004). The study of wild relatives of model species, however, can be very useful because they show adaptations to specific environments that are not necessarily encountered by model species (Mitchell-Olds 2001). Tolerance to elevated concentrations of heavy metals has been developed by a small number of species in response to highly contaminated soils and provides an excellent model system to study adaptive evolutionary processes to extreme environments (Macnair 1993). Heavy metals sometimes occur at elevated soil concentrations either through natural processes, as in nickel-rich serpentine soils, or through human activities. The development of mining or smelting activities in the 19th century left soils highly contaminated by zinc (Zn), cadmium (Cd), lead (Pb) and copper (Cu) in many locations (Macnair 1993). Some of these heavy metals, like Zn and Cu, are oligo-elements that are essential in small quantities for normal plant development. The total metal content of contaminated sites can, depending on the metal, be up to 10- to 1000-fold higher than

that of uncontaminated sites (Bert *et al.* 2002). At these extreme concentrations, both essential and nonessential heavy metals become toxic (Clemens 2001; Hall 2002).

Arabidopsis halleri, which is together with *A. lyrata* the closest wild relative of *A. thaliana* (Mitchell-Olds 2001), has developed high Zn and Cd tolerance (Bert *et al.* 2000). Surprisingly, in *A. halleri* high Zn tolerance is not restricted to metallicolous populations but has also been reported for non-metallicolous populations, which might indicate that Zn tolerance appeared in the species long before the occurrence of heavy metal contaminated soils following the industrial revolution. However, the recent colonization of these industrial polluted sites by *A. halleri* individuals from the geographically closest non-metallicolous populations (Pauwels *et al.* 2005) has probably involved an increase in Zn tolerance, as observed in controlled conditions for one metallicolous population compared to one growing in unpolluted soil (Bert *et al.* 2000).

Two main strategies have been applied during the past decades to identify the precise genetic mechanisms underlying heavy metal tolerance. Since heavy metal tolerance is thought to have appeared through adaptations of metal homeostasis processes including metal uptake, chelation, trafficking and storage (Hall 2002), a first strategy has been to identify and subsequently investigate metal homeostasis genes. A direct correlation between metal tolerance and the synthesis of specific chelators has been established only for Ni in the Ni-hyperaccumulating plant *Alyssum lesbiacum* and for arsenate (As) in the As-tolerant grasses *Holcus lanatus* and *Agrostis castellana* (Krämer *et al.* 1996; Hartley-Whitaker *et al.* 2001; Schat *et al.* 2002). An alternative strategy, involving a global comparison of *A. halleri* and *A. thaliana* through transcription profiling, has been applied recently to identify genes differentially expressed and/or differentially regulated in *A. halleri* compared to *A. thaliana* under various metal conditions. These studies identified *A. halleri* homologues of *A. thaliana* genes that are potentially involved in Zn tolerance (Becher *et al.* 2004; Weber *et al.* 2004). However, differential expression and/or regulation may just as well be the primary cause of heavy metal tolerance as its consequence, or might simply be the result of the divergence time separating *A. halleri* and *A. thaliana*. Thus, definitive evidence

for their implication in heavy metal tolerance is still missing. Until recently, the constitutive nature of Zn tolerance in *A. halleri* rendered its genetic analysis inaccessible. Macnair et al. (1999) circumvented this major handicap by analysing the segregation of Zn tolerance in an F2 population produced by interspecific crosses performed between the tolerant *A. halleri* and its closest non-tolerant relative *A. lyrata* ssp. *petraea*. Based on the segregation analysis of one F2 progeny, the authors hypothesized a single major gene determining Zn tolerance in *A. halleri*, already described for other metals and species (Schat *et al.* 1993; Smith and Macnair 1998). Furthermore, circumstantial evidence was recently provided in favour of a role of the cation diffusion facilitator protein MTP1 in Zn tolerance in *A. halleri* given its co-segregation with Zn tolerance in a backcross 1 progeny of an interspecific cross between *A. halleri* and *A. lyrata* ssp. *petraea* (Dräger *et al.* 2004).

Quantitative trait loci (QTL) mapping has proved to be very powerful in examining complex adaptive traits (Doebley *et al.* 1997; Alonso-Blanco *et al.* 1998; Ungerer *et al.* 2002; Weinig *et al.* 2003); it provides an efficient means to determine the number of genes implicated in a trait as well as their effects and interactions, which are important to understand the evolutionary history of a trait (Mackay 2001; Erickson *et al.* 2004). By identifying specific chromosomal regions where genetic variation can be associated with measurable phenotypic variation (Tanksley 1993; Doerge 2002), QTL mapping can help to detect or validate candidate genes underlying complex traits (Flint and Mott 2001; Yano 2001; Glazier *et al.* 2002). The application of a QTL approach in *A halleri* was highly enhanced by the recent publication of the genetic linkage maps of its close relatives *A. l. petraea* and *A. l. lyrata* (Kuittinen *et al.* 2004; Yogeeswaran *et al.* 2005). The extensive conservation of marker order reported between *A. lyrata* subspecies and the model *A. thaliana* (Kuittinen *et al.* 2004; Yogeeswaran *et al.* 2005) made the prospect of transferring these resources to *A. halleri* even more attractive.

In this study we have applied a QTL approach to investigate the genetic basis underlying Zn tolerance in *A. halleri*. We performed an interspecific cross between *A. halleri* and *A. l. petraea* to generate a first generation backcross (BC1). This progeny, segregating for Zn tolerance, was used to construct a molecular linkage map (the first

reported for a cross between these two species) and to identify QTL regions for Zn tolerance in *A. halleri*, making full use of the previous mapping experiments conducted on *A. lyrata* subspecies.

4.2 Materials and methods

Plant material

A single cross was performed between one individual from the Zn-tolerant species *A. halleri* ssp. *halleri* (henceforth called *A. halleri*) (pollen donor) and one from the non-tolerant species *A. lyrata* ssp. *petraea* (*A. l. petraea* 1) (pollen recipient). The *A. halleri* individual (*2n* = 16) originated from a site highly contaminated with Zn, Cd and Pb (Auby, France) (Van Rossum *et al.* 2004). The *A. l. petraea* 1 individual (*2n* = 16) originated from an uncontaminated site in the Czech Republic (Unhost, Central Bohemia) (Macnair *et al.* 1999). Both species are self-incompatible and usually outcrossing. One randomly selected F1 individual was used as male parent to fertilize a second *A. l. petraea* individual (*A. l. petraea* 2), generating the interspecific backcross progeny (BC1). The BC1 population used for linkage map construction and QTL mapping consisted of 199 individuals.

Evaluation of Zn tolerance

Twelve replicates of the four parental genotypes and three replicates of each of the BC1 individuals were obtained by vegetative propagation. They were grown in the greenhouse on sand, and 8 weeks after cloning transferred to 10-liter polycarbonate trays containing a nutrient solution. The plants were randomly arranged in the trays (48 plants per vessel, including one copy of each parental genotype), which in turn were randomized in the growth chamber twice a week. The nutrient solution consisted of 0.5 mM $Ca(NO_3)$, 0.2 mM $MgSO_4$, 0.5 mM KNO_3, 0.1 mM K_2HPO_4, 0.2 µM $CuSO_4$, 2 µM $MnCl_2$, 10 µM H_3BO_3, 0.1 µM MoO_3, 10 µM FeEDDHA and 10 to 3000 µM Zn added as $ZnSO_4$.The pH of the solution was set at 6.5.

Zn tolerance was measured by a sequential test established by Schat and Ten Bookum (1992). This test provides a measure for tolerance by sequentially transferring plants into increasing concentrations of zinc and determining for each individual the lowest concentration at which no new root growth is produced (the EC100). Roots of plants were blackened with activated charcoal to observe new root growth more easily. The plants were grown on 10 μM of Zn for the first three weeks. After verification of their root growth at 10 μM, 6 to 12 copies of each parental genotype and one to 3 copies of each BC1 individual were transferred in successive weeks to 25, 50, 75, 100, 150, 250, 500, 1000, 2000 and 3000 μM of Zn. Root growth of the plants was evaluated at the end of each week. Plants were observed for at least two weeks after reaching their EC100, to ensure that no new root growth occurred.

Marker analysis

Genomic DNA of the four parental genotypes and of the 199 individuals of the BC1 was extracted for marker analysis using a slightly modified Dellaporta method (Saumitou-Laprade *et al.* 1999).

The BC1 progeny was genotyped using 65 sequence-based markers anchored in *A. thaliana* and 19 AFLP markers (Table 4.1). The anchored markers consisted of microsatellites, indels and polymorphisms revealed by CAPS-, RFLP- and SSCP-analysis. Some markers were selected because of their potential implication in metal homeostasis (*FRD3*, *HMA4*, *MTP1*, *MTP3*, *NRAMP3*, *NRAMP4* and *MT2B*). Others had been identified by transcription profiling analyses conducted on *A. halleri* (*At1g46768*, *HMA4*, *NRAMP3*, *At2g40140*, *At2g43010*, *MTP1*, *At3g28220*, *At4g33160*, *At4g38220* and *At5g08160*) (Becher *et al.* 2004; Weber *et al.* 2004) (A. Radu Craciun, personal communication) (S. Mari, personal communication). The remainders were selected using their position in the *A. thaliana* genome, to improve coverage of the *A. halleri* genome. Thirty-eight of the 65 anchored markers were previously reported for *A. thaliana* and/or *A. l. petraea* (Bell and Ecker 1994; Clauss *et al.* 2002; Kuittinen *et al.* 2002; Kuittinen *et al.* 2004); 31 of the 38 markers had already been mapped in *A. l. petraea* (Kuittinen *et al.* 2004). In addition, 27 newly defined markers were introduced in this study. For 15 of the 27 markers, primer design and/or genotyping of the BC1

progeny were kindly performed by colleagues (Table 1). For the other 12 markers, we designed primers on the basis of the *A. thaliana* sequence (TAIR database at http://www.arabidopsis.org/) using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). For four of these 12 markers (*At2-TCA1*, *At2-TA5*, *At4-GA2* and *At4-TC1*), primer pairs were designed to amplify small fragments of 150 to 400 base pairs (bp). For the other 8 markers (*At1g46768*, *At2g43010*, *At3g28220*, *At3g33530*, *At3g45810*, *At5g08160* and *At4g38220*), one primer was designed in an exonic region of the gene and the second primer was placed either in the following exon or in the 5'-upstream region of the start codon. In the latter case the possible promoter region of the gene, supposed to be highly variable between species, was targeted. For amplification of *EMF2*, already mapped in *A. l. petraea,* new primer sequences were designed as described above and used instead of the ones defined by Kuittinen *et al.* (2004). In order to obtain labelled PCR products detectable on the automated genotyper Li-Cor 4200 (Li-Cor-ScienceTec), either the forward or reverse primer contained a 5'-tail of 19 bp (forward primer) or 20 bp (reverse primer) homologous to the universal consensus M13 primer sequence, followed by the locus-specific sequence (Oetting *et al.* 1995). The following polymerase chain reaction (PCR) conditions were applied for all 65 markers, except for those that were provided through collaborations (Table 1). PCR reactions were carried out in a total volume of 15 µl containing 20 ng of template DNA, 2 mM $MgCl_2$, 0.2 mg/ml BSA, 0.2 mM dNTP, 0.2 µM of each unlabelled primer and 0.15 µM of the M13 fluorescently labelled primer (either IRD-700 or IRD-800), 20 mM Tris-HCl (pH 8.3), 50 mM KCl, and 0.4 units of AmpliTaq® DNA Polymerase (Applied Biosytems). PCR was performed on a Perkin-Elmer Gene-Amp system 9700 (94° for 5 min, followed by locus-specific amplification: 94° for 30 s, annealing temperature for 45 s, 72° for 40 s, for eight cycles, followed by M13 labelling amplification: 94° for 30 s, 50 °C for 20 s, 72° for 40 s, for 30 cycles, and a final extension 72° for 7 min). The annealing temperature of the locus-specific cycles varied between 50 and 60°, depending on the locus. For CAPS markers, restriction was carried out in a total volume of 20 µl containing 10 µl of PCR product, 0.2 mM spermidine, 1x specific enzyme buffer provided by the supplier and 1 unit of restriction enzyme. Restriction was carried out by incubation for at least 4 hr at the appropriate temperature in a Perkin-Elmer

Gene-Amp system 9700. Polymorphisms were revealed on agarose or polyacrylamide gels using a Li-Cor genotyper.

TABLE 4.1 Markers used in linkage map construction.

Locus name	Identification/ BAC location in *A. thaliana*	Type of polymorphism	Forward primer	Reverse primer
			Linkage group 1	
AthACS	F22L4	Microsat	b	b
AAC/CG-88		AFLP		
AXR1	At1g05180	Indel	e	e
PhyA	At1g09570	Indel	e	e
ICE10	F12F1	Microsat	b	b
ICE13	At1g13220	Microsat	b	b
GI	At1g22770	Indel	e	e
AGG/CT-198		AFLP		
ATTS0392	At1g30630	Microsat	b	b
ACA/CG-228		AFLP		
VIP1	At1g43700	Indel	e	e
At1g46768	At1g46768	Indel	AGGTTGATCATTTTCTAAAAGTTCTTG	TTCCTCCTCCGTATCCCTCT
MTP1-C		RFLP/SSCP	d	d
			Linkage group 2	
F19K23	At1g62050	Microsat	b	b
ACA/CG-87		AFLP		
ACA/CG-160		AFLP		
ACA/CG-320		AFLP		
AAC/CG-84		AFLP		
SLL2	At1g66680	Indel	e	e
lyr132[d]	T23K23	Microsat	GCCGTGAGATTAAAGAAGACG	GCAAGAGCTGATCTCCATCC
ACA/CG-71		AFLP		
nga111	F28P22	Microsat	a	a
At1g75830[c]	At1g75830		CATATCTATGCAAATTGTGTTTAATATA	ACATGGGAAGTAACAGATACACTTATGA
ADH1	At1g77120	Indel	e	e
			Linkage group 3	
At3g08040[b]	At3g08040	Indel	TACCAACCAGCCACAGCAACC	CGCTTTGTTTCCACTATTTGACTTTG
MDC16	MDC16	Microsat	b	b
nga162	MDC16	Microsat	a	a
DMC1	At3g22880	Indel	e	e
At2-TCA1	T9F8	Microsat	CAACCACACCCCTTTAGCTT	GAGAGCCCATGGAGATGAAG
HMA4	At2g19110	Indel	TGACCTGAAAATGAAAGGTGGTC	TGCATAACTCCTGCAACAGCT
ICE14	F11A3	Microsat	a	a
ACA/CG-310		AFLP		
			Linkage group 4	
con	At2g21320	MseI	e	e
At2g22430[g]	At2g22430	Indel	AGTTCAGATTCAGTGGGTGG	GTAGATCTGTGAAACTCCGG
Ck2-alpha2	At2g23080	Indel	e	e
NRAMP3[e]	At2g23150	PsiI	TTGGATGTTTGGTCAAGCTAAGCCAAGTG	TGCCACGAGCAATGAGGTAGAGGATGAAT
At2-TA5	T19L18	Microsat	TCATCGGATCCATATTTGTTTG	CATTGTTGGTCGTGGCTATG
ELF3	At2g25930	Microsat	e	e
At2g28700[g]	At2g28700	Indel	TGGGAACATGGGAGATTTTGTTATG	TGTCTTGCCCTTCACTGAAAAAGAG
nga361	T16B12	Microsat	a	a
At2g33010[g]	At2g33010	Indel	TGGAGCATATCGAGAAGAAGCACTC	GGACTTCTTGTGAAGGCAAATCAGA

TABLE 4.1 (continued)

Locus name	Identification/ BAC location in *A. thaliana*	Type of polymorphism	Forward primer	Reverse primer
ACT3	At2g37620	MspI	e	e
ICE12	At2g39010	Microsat	a	a
At2g40140	At2g40140	Indel	g	g
At2g43010	At2g43010	Indel	CGGACTCATGGACTTGCTTT	TTCTGGGTTTGGGTTTGTTC
ACA/CG-130		AFLP		
ICE11	F11C10/F13A10	Microsat	a	a
MTP1-A	At2g46800	RFLP/SSCP	d	d
			Linkage group 5	
nga1145	T16F16	Microsat	a	a
At3g28220	At3g28220	Indel	TGGGTCCATTTCTTGTTGTT	CCAAGCCAATTGCTCCATAG
At3g33530	At3g33530	MfeI	TGGTGGGATGTAACAACAGG	TTTAGACTGGGGCACAAAGC
AAC/CG-179		AFLP		
At3g45810	At3g45810	DraI	TTTGCTGGTTATTGCCTACG	ACCTCTCGCTCTTGTTTCCA
F3H	At3g51240	HindIII	c	c
MTP3[b]	At3g58060	Indel	CCATGGTCACGGTCATAGTCAT	CGCTCTGTATCGAATCTCCCAGCA
nga112	At3g62650	Microsat	a	a
nga1145	T16F16	Microsat	a	a
			Linkage group 6	
At4-GA2	T18A10	Microsat	CCTCGGGTAAAGACAGAGCA	TGGTAACACCGGAAGTTTCA
LD	At4g02560	Indel	e	e
MTP1-B		RFLP/SSCP	d	d
AthDET1	At4g10180	Microsat	b	b
ICE2	K18P6	Microsat	b	b
AthCDPK9	MQM1	Microsat	b	b
AAC/CG-336		AFLP		
ACA/CG-359		AFLP		
At5g08160	At5g08160	Indel	TTGTTGGTACGACTTTTTCTCG	GCCGGATCCTTGACTTTCTT
MT2b	At5g02380	RFLP	f	f
			Linkage group 7	
At4g38220	At4g38220	Indel	GTGGTGGTGGGAGAAGAGAG	ACCGTGTTGATTCGGAGGTA
At4g33160	At4g33160	Indel	g	g
cha15[f]	F11C18	Microsat	AATTCAAACAGGCGAAACCA	CTGCGAATCTCACGACTTCA
TSB2	At4g27070	Indel	e	e
At4-TC1	T19F6	Microsat	CAAGGTCGAATGTTGGAGACT	GCGCACTACAAAAATGAGAGG
SRK[a]	At4g21366	SNP	TCAAGATTGAAGCTGAGTGA	TACACAACCCGTCCCGCCAA
FCA	At4g16280	HinfI	c	c
ACA/CG-266		AFLP		
ACA/CG-312		AFLP		
PhyC	At5g35840	Indel	c	c
ATTSO191	At5g37780	Microsat	b	b
ICE9	At5g40340	Microsat	b	b

TABLE 4.1 (continued)

Locus name	Identification/ BAC location in *A. thaliana*	Type of polymorphism	Forward primer	Reverse primer
			Linkage group 8	
ATCLH2	At5g43860	Indel	e	e
ACA/CT-86		AFLP		
EMF2	At5g51230	XhoI	GTTGCAGTTTGCAAAAACGA	CATGGAATGTGACCATCTGC
ACA/CG-387		AFLP		
MHJ24	MHJ24	Microsat	b	b
NRAMP4[e]	At5g67330	Indel	TTGGATGTTTGGTCAGACGAAACCCAGTG	ATAAACTGTCCGGCGTACGTACCTGTGAT

The names and identification of the loci in *A. thaliana*, the type of polymorphism scored, restriction enzyme if CAPS marker, primer sequences if newly defined markers. Reference is given if primers are described elsewhere: (a) Bell and Ecker (1994), (b) Clauss *et al.* (2002), (c) Kuittinen *et al.* (2002), (d) Dräger *et al.* (2004), (e) Kuittinen *et al.* (2004), (f) Zhou and Goldsbourgh (1995), (g) A. Radu Craciun, pers. comm.

[a]*A. halleri* allele-specific primers are given, genotypes have been controlled by amplification with *A. l. petraea* 1 allele-specific primers (V. Castric, pers. comm.).

[b]primers and genotyping results provided by M. Hanikenne (pers. comm.).

[c]primers and genotyping results provided by M. Mirouze (pers. comm.).

[d]primers provided by T. Mitchell-Olds (pers. comm.).

[e]primers and genotyping results provided by R. Oomen (pers. comm.).

[f]primers provided by C. Schlötterer (pers. comm.).

[g]primers and genotyping results provided by F. Varoquaux (pers. comm.).

Amplified Fragment Length Polymorphism (AFLP) marker analysis was performed as described by Vos *et al.* (1995), using *Eco*RI/*Mse*I restriction enzymes. For pre-amplification, one nucleotide was added to *Eco*RI and *Mse*I. For selective amplification, 3 and 2 nucleotides were added to *Eco*RI and *Mse*I respectively. The *Eco*RI selective primer was fluorescently labelled with either IRD-700 or IRD-800 for visualisation of the AFLP bands on a Li-Cor genotyper 4200 (Li-Cor-ScienceTec). Polymorphic and segregating bands were scored using the program RFLPSCAN 3.0 (Scanalytics). Their sizes were determined by comparison with an appropriate labelled molecular weight marker (50-700 bp, Li-Cor-ScienceTec). AFLP markers were named according to the selective nucleotides used in selective amplification and their size.

Linkage map construction

The *A. halleri* x *A. l. petraea* (*Ah* x *Alp*) linkage map was constructed with the Joinmap 3.0 program (Van Ooijen and Voorrips 2001). Individuals lacking information

for more than 25% of all markers were excluded from the analysis. Linkage groups were obtained at a logarithm-of-odds (LOD) score threshold of 4. Markers along each linkage group were ordered using the sequential method implemented in Joinmap. In this method, the best order was determined by comparing the goodness-of fit of the resulting map for each tested order using a threshold of 0.5 and 1.0 for the linkage groups and the loci respectively. Kosambi's mapping function was used to translate recombination frequencies into map distances (Kosambi 1944).

Linkage map analysis

We performed a t-test for correlated samples (Minitab, State College, PA) to test for a significant difference in marker intervals between the *Ah* x *Alp* and the *A. l. petraea* maps using the markers common to both mapping experiments. A t-test for correlated samples (Minitab, State College, PA) was also performed to compare the linkage group lengths in the *Ah* x *Alp* and either the *A. l. petraea* or the *A. l. lyrata* maps.

Marker segregation

According to Mendelian inheritance, the *A. halleri* alleles are expected to segregate in a 1:1 ratio in the BC1. When dealing with an interspecific cross, segregation distortion frequently occurs. Deviations from the Mendelian ratios were tested using a chi-square test implemented by Joinmap 3.0 at a locus-by-locus significance level $\alpha = 0.05$ (Van Ooijen and Voorrips 2001).

Statistical analysis and QTL mapping

We performed a Kruskal-Wallis test, based on wilcoxon rank scores of the data, to test for significant differences of Zn tolerance among the four parental genotypes using the NPAR1WAY procedure in SAS (SAS 1999). A one-way analysis of variance (ANOVA) using the GLM procedure in SAS (SAS 1999) was performed on the EC100 values obtained for the replicates of the 199 BC1 individuals to determine the genotype effect. The broad-sense heritability of zinc tolerance was calculated by dividing the genetic variance by the total phenotypic variance, using the mean square values (MS) from the ANOVA ($h^2 = MS_{genot} / (MS_{genot} + MS_{error})$. Type III sums of squares were

used because the data set was unbalanced, due to an unequal number of clones of each BC1 individual.

The QTL analysis for Zn tolerance was performed on the $EC100_{mean}$ values (the arithmetic mean of the EC100 values of the clones) of the BC1 genotypes using the MapQTL 4.0 program (Van Ooijen *et al.* 2002). The LOD score threshold for QTL detection was set at 2.3 ($\alpha = 0.05$) and obtained by a permutation test on the quantitative data in MapQTL. A first QTL analysis was performed using interval mapping (IM) as implemented in MapQTL. The LOD score representing the likelihood of a QTL being present has been calculated every centiMorgan (cM) within the intervals along the linkage groups. Markers for which the LOD score exceeded the significance threshold were identified in each linkage group. Automatic cofactor selection was performed by MapQTL on these markers for their use as cofactors in MQM analysis. We performed MQM mapping twice while adjusting the selection of the cofactors to obtain the best possible set of QTLs, i.e. showing maximal LOD scores. One- and two-LOD support intervals were obtained using Mapchart 2.1 (Voorrips 2002). The estimated additive genetic effect (*a*) and the percentage of variance explained by each QTL (R^2) were calculated in IM. We tested for significant interactions between QTLs using the GLM procedure in SAS (SAS 1999). The markers closest to or at the QTL position were considered as random factors.

4.3 Results

Linkage map construction

Of the 199 BC1 individuals, 196 individuals could be genotyped successfully at more than 75% of all markers and were used for the map construction. A total of 86 markers were assigned to eight linkage groups (LG1-LG8) using a LOD score threshold of 4 (Table 4.1). The lengths of the linkage groups varied from 57 to 80 cM and summed to a total of 567 cM. The average distance between two adjacent markers was 6.6 cM, ranging from 1 to 27 cM (Figure 4.1).

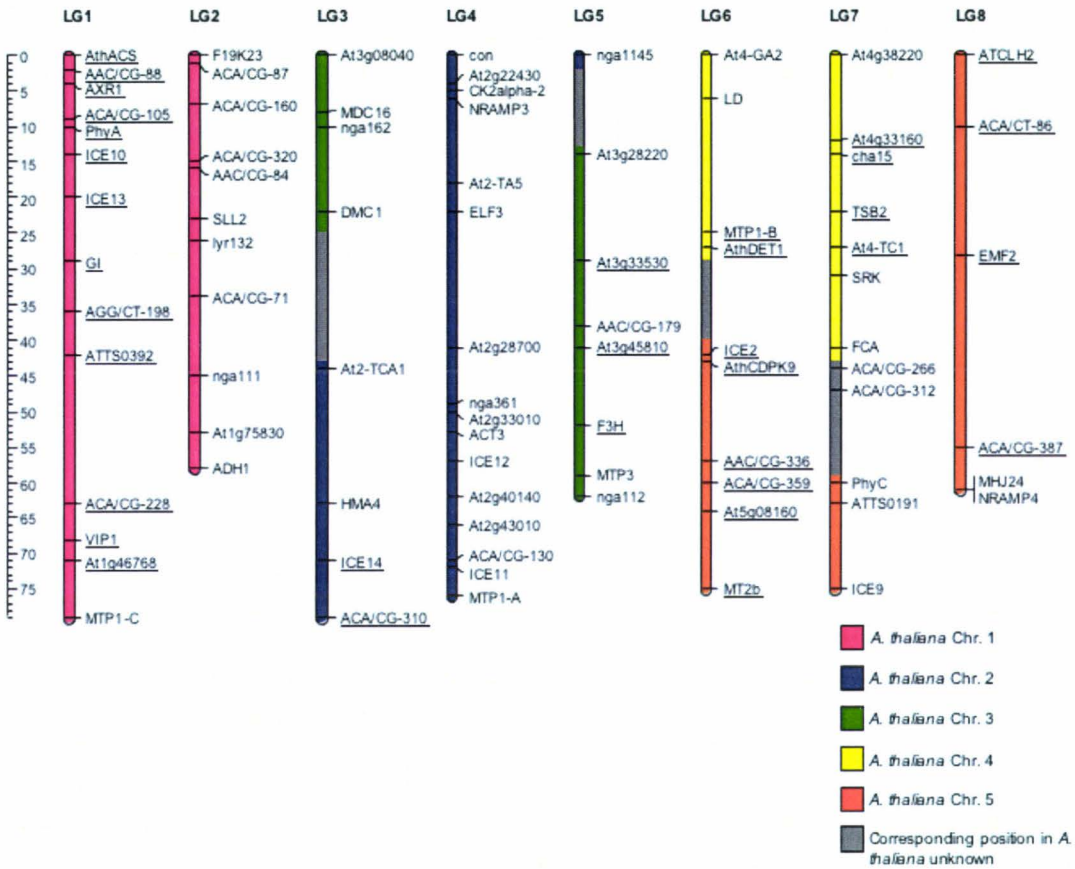


FIGURE 4.1 Linkage map of the *A. halleri* x *A. l. petraea* BC1 progeny constructed with Joinmap 3.0.

The homology with the *A. thaliana* chromosomes is indicated by the colors. The regions where translocation occurred are indicated in gray. Position in *A. thaliana* of the anonymous markers was inferred by integration of the *A. l. petraea* and *A. l. lyrata* mapping experiments. Markers in segregation distortion are underlined.

Comparative analysis of the *A. halleri* x *A. l. petraea* map with the maps of *A. l. petraea* and *A. l. lyrata*

The transition from eight chromosomes in the *Ah* x *Alp* map to five chromosomes in the *A. thaliana* genome can be explained by five main chromosomal rearrangements as described for *A. l. petraea* (Kuittinen *et al.* 2004; Koch and Kiefer 2005) and *A. l. lyrata* (Yogeeswaran *et al.* 2005). These consist of three fusions between the linkage groups LG1/LG2, LG3/LG4 and LG7/LG8 and two reciprocal translocations between LG3/LG5 and LG6/LG7 (Figure 4.1). Marker order in the *Ah* x *Alp* and *A. thaliana* maps was generally similar. The order of the 31 marker loci shared by the *Ah* x *Alp* and

A. l. petraea linkage maps was identical, with one exception on LG1. The positions in the interspecific map of the loci (*PhyA* and *AXR1*) did not agree with those reported for *A. l. petraea,* but rather with those expected from *A. thaliana.*

The marker distances obtained on the marker intervals between the markers common to the *Ah* x *Alp* and *A. l. petraea* maps were not significantly different in both mapping experiments (P = 0.27). The linkage group lengths differed significantly between the *Ah* x *Alp* map and either the *A. l. petraea* (P = 0.03) or the *A. l. lyrata* map (P = 0.02) (Table 4.2).

TABLE 4.2 Comparison of linkage group lengths in the *A. halleri* x *A. l. petraea*, the *A. l. petraea* and the *A. l. lyrata* maps

Linkage group	*A. halleri* x *A. l. petraea*[a]	*A. l. petraea*[a]	*A. l. lyrata*[a]
LG1	78	74	39
LG2	57	58	6
LG3	80	64	69
LG4	76	67	47
LG5	63	54	60
LG6	75	61	76
LG7	76	78	49
LG8	62	59	61
Mean linkage group length	70.9	64.4	50.9

[a]Length of the linkage groups are given in cM.

Two duplication events of the *MTP1* gene (a single-copy gene on *A. thaliana* chromosome 2 and *A. lyrata* LG4) were detected in *A. halleri*. We mapped the three copies identified in *A. halleri* (*MTP1-A*, *MTP1-B* and *MTP1-C*) (Dräger *et al.* 2004) on three different linkage groups (LG4, LG6, and LG1 respectively). The *A. halleri MTP1-A* copy mapped to the lower arm of LG4 beyond the marker *ICE11* (the expected position from *A. thaliana*) and can therefore be considered as the ortholog of the *A. lyrata* and *A. thaliana MTP1* gene.

Markers in segregation distortion

At a locus-by-locus significance level of 0.05, 34 markers (40%) showed distorted segregation and were found on six of the eight linkage groups (Figure 4.1). The segregation ratio bias was highly directional. Of the 34 distorted markers, 31 showed an excess of the *A. l. petraea* 1/*A. l. petraea* 2 homospecific (i.e. originating

from the same species) allelic combination compared to the *A. halleri*/*A. l. petraea* 2 heterospecific (i.e. originating from different species) genotype. Only three markers, all located on linkage group LG5 showed the opposite pattern, *i.e.* an excess of the heterospecific combination. With a single exception (on LG5) distorted markers were always linked to markers distorted in the same direction, indicating that the segregation bias was due to meiotic events rather than genotyping errors.

Evaluation of Zn tolerance

A Kruskal-Wallis test showed a highly significant difference among the tolerance levels of the four parental lines[4] of the BC1 ($P < 0.0001$). Pairwise comparisons revealed a significant difference in Zn tolerance between the *A. halleri* parental clones (n = 12; $EC100_{mean}$ = 2917 µM Zn) and the *A. l. petraea* 1 parental clones (n = 6; $EC100_{mean}$ = 38 µM Zn) ($P = 0.0001$). The Zn tolerance of the *A. halleri* parental clones was probably underestimated because even at the highest concentration applied in the test (3000 µM Zn), all clones except one still showed new root growth. The Zn tolerance of the F1 parental clones (n = 12; $EC100_{mean}$ = 1708 µM Zn) differed significantly from the tolerance of the *A. l. petraea* 1 ($P = 0.0005$) and *A. halleri* parental clones ($P < 0.0001$). This indicates partial dominance of Zn tolerance in *A. halleri*, even though the underestimation of Zn tolerance for the *A. halleri* parental clones precludes any estimation of the dominance coefficient. No significant difference was identified between Zn tolerance of *A. l. petraea* 1 and *A. l. petraea* 2 parental clones (n = 9; $EC100_{mean}$ = 67 µM Zn) ($P = 0.2207$). The genotype effect of Zn tolerance[5] of the BC1 individuals was highly significant ($F = 2.22$; $P < 0.0001$). Broad-sense heritability of Zn tolerance in the BC1 was high ($h^2 = 0.69$). $EC100_{mean}$ values showed a bimodal distribution in the BC1 (Figure 4.2). No transgressive segregation of Zn tolerance was observed.

[4] See Annexes TABLE 1 for EC100 values
[5] See Annexes TABLE 1 for EC100 values

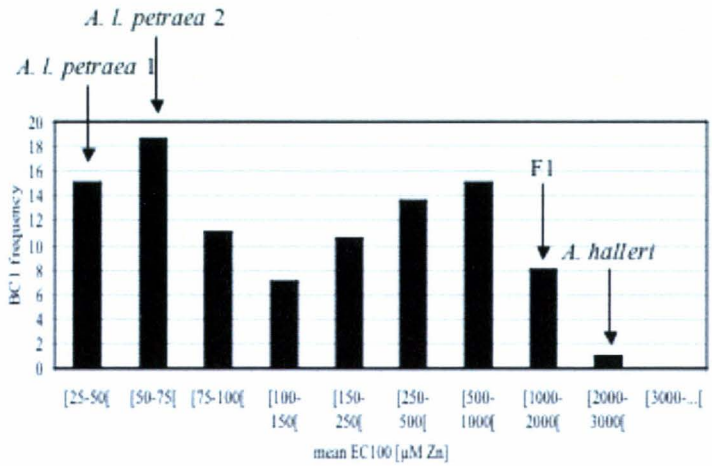


FIGURE 4.2 Frequency distribution of Zn tolerance in the BC1 derived from *A. halleri x A. l. petraea.*

Zn tolerance of 199 BC1 individuals and the parental genotypes was measured by a sequential Zn exposure test in hydroponic solution. The Zn tolerance means of the parental genotypes (*A. halleri, A. l. petraea* 1, F1 and *A. l. petraea* 2) are indicated above the graph.

QTL mapping of Zn tolerance

Three QTLs located on linkage groups LG3, LG4 and LG6 were identified by IM and subsequent MQM mapping (Figure 4.3; Figure 4.4). The QTLs were named Zntol-1, -2 and -3, respectively for linkage group LG3, LG4 and LG6. LOD scores of Zntol-1, -2 and -3, calculated by the MQM module of MapQTL, were 6.46, 7.28 and 4.52 respectively. On the five other linkage groups the LOD scores did not exceed the significance threshold value of 2.4.

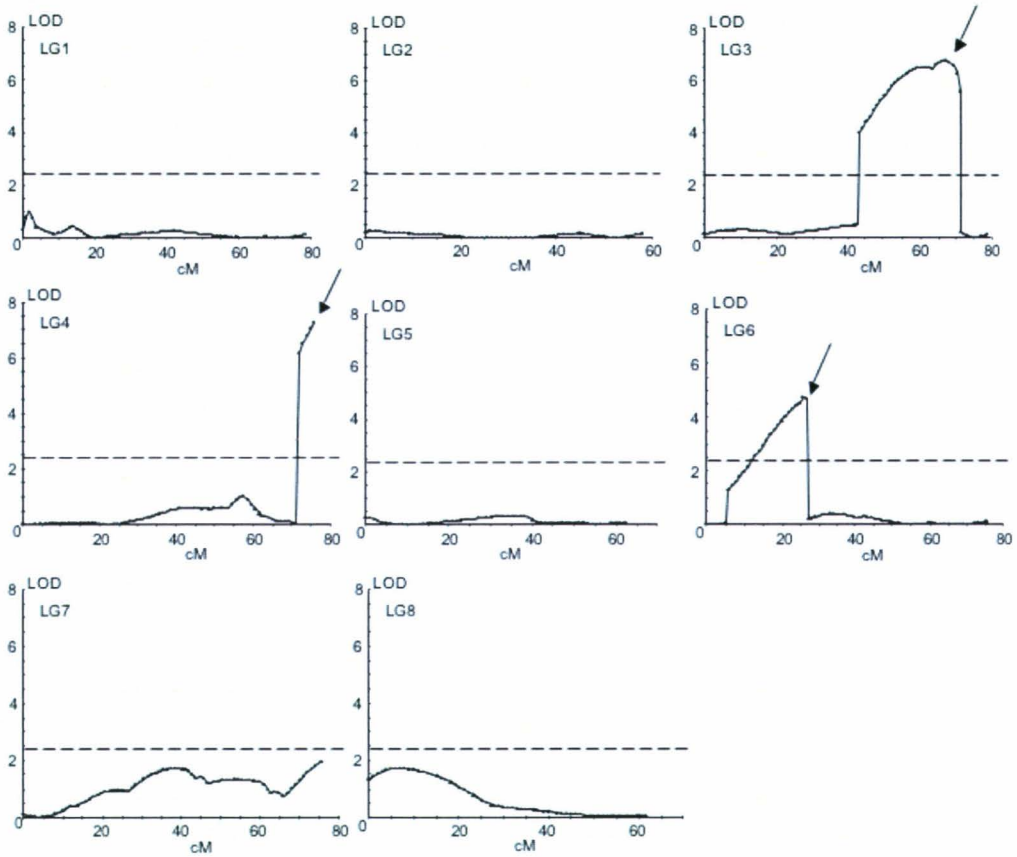


FIGURE 4.3 LOD score profiles for Zn tolerance.

Names of linkage groups are given at the left corner of each graph. Map positions are plotted along the abscissa. LOD scores are plotted along the Y-axis. Dashed lines correspond to the LOD score threshold (2.3) for QTL detection at an error level α = 0.05. QTLs are indicated by arrows above the LOD score profile.

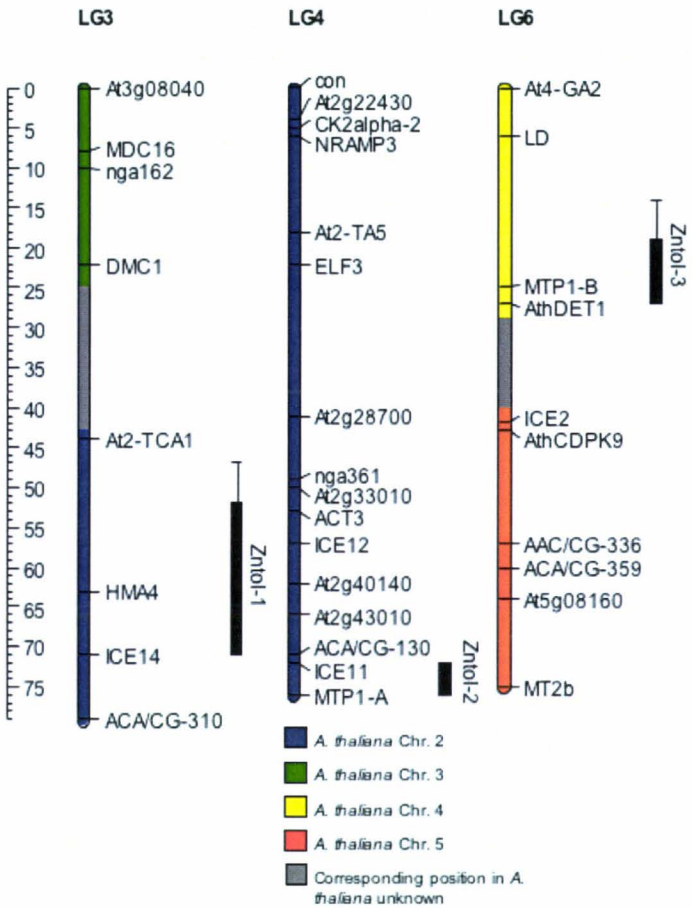


FIGURE 4.4 LOD support intervals for the QTLs for Zn tolerance in the BC1 progeny.

The position of a QTL is shown as the interval over which the LOD score is within 1 or 2 log-units of its maximum value, i.e. at the most likely position of the QTL. Bars indicate 1-LOD (10-fold) support intervals and whiskers (lines extending beyond bars) indicate 2-LOD (100-fold) support intervals. The homology with the *A. thaliana* chromosomes is indicated by the colors.

The individual contribution of each QTL to the phenotypic variance was 12.2%, 11.2% and 5.6% respectively for the QTLs Zntol-1, -2 and -3. They accounted together for 29% of the total phenotypic variance, which represents 42% of the genetic variance. For each of the QTLs, the *A. halleri* allele increased Zn tolerance (Table 4.3). Pairwise interactions between the QTLs were significant at $\alpha = 0.05$ (Table 4.4). For Zntol-1, Zntol-2 and Zntol-3 one-LOD support intervals of 19 cM, 4 cM and 8 cM were reported. Two-LOD support intervals were 24 cM, 4 cM and 13 cM for the three QTLs respectively (Figure 4.4). Six markers co-localised with the LOD support intervals. Markers *ICE14* and *HMA4* mapped in the LOD support interval of Zntol-1, markers

MTP1-A and *ICE11* co-localised with Zntol-2, and markers *AthDET1* and *MTP1-B* co-localised with Zntol-3.

TABLE 5.3 QTLs for Zn tolerance

QTL[a]	LG- Marker[b]	Position[c]	LOD Score[d]	R^{2e}	a[f]
Zntol-1	LG3-HMA4	64.6	6.46	12.2	-280.861
Zntol-2	LG4-MTP1-A	75.5	7.28	11.2	-266.055
Zntol-3	LG6-MTP1-B	24.7	4.52	5.6	-197.275

[a]QTLs are named by the trait and ordered from 1 to 3.
[b]Linkage group on which QTLs were mapped and marker at QTL position or closest to QTL position.
[c]Position of marker at or closest to QTL (centiMorgans).
[d]LOD score of marker at or closest to QTL.
[e]% of explained variance of the marker at or closest to QTL.
[f]Additive effects are given as the difference between the means of the two genotypic groups of BC1 individuals (negative value implies that the *A. halleri* allele increases Zn tolerance compared to the *A. l. petraea* allele).

TABLE 5.4 Epistatic interactions between QTLs for Zn tolerance

QTL x QTL interaction[a]	P[b]
Zntol-1 x Zntol-2	0.0306
Zntol-1 x Zntol-3	0.02
Zntol-2 x Zntol-3	0.0482

[a]Pairwise interactions between QTLs for Zn tolerance.
[b]Significance levels of interactions.

4.4 Discussion

Previous combinations of classical functional and transcriptional analyses have identified several genes potentially involved in the adaptation of *A. halleri* to high heavy metal concentrations. However, definitive evidence for their implication in heavy metal tolerance has not yet been provided, and the genetic mechanisms underlying this trait are still largely unknown. This paper contributes to a better understanding of the genetic architecture of Zn tolerance by applying a QTL mapping approach to the Zn tolerant species *A. halleri*. The linkage map, constructed using an *A. halleri* x *A. l. petraea* BC1 progeny, revealed three QTL regions determining Zn tolerance, in which

we would expect genes involved in the adaptation of *A. halleri* to high heavy metal concentrations to be located.

Linkage map analysis

The extensive conservation of marker order between the interspecific *Ah* x *Alp* map and *A. thaliana* was in line with previous results reported for the *A. l. petraea* (Kuittinen *et al.* 2004; Koch and Kiefer 2005) and *A. l. lyrata* (Yogeeswaran *et al.* 2005) maps. The only discrepancy in marker order between the *A. l. petraea* and the interspecific map was observed for the loci *PhyA* and *AXR1* (LG1/Al1), but as suggested by Kuittinen et al. (2004) their order in *A. l. petraea* "could well be consistent with the order expected from *A. thaliana*". The large inversion observed on Al6 of the *A. l. petraea* linkage map (Kuittinen *et al.* 2004), and confirmed in *A. l. lyrata* (Yogeeswaran *et al.* 2005) as well as in another close relative *Capsella rubella* (Boivin *et al.* 2004) was not detected on the corresponding linkage group LG6 on the *Ah* x *Alp* map. Nevertheless the low marker density in this region probably precluded the detection of this inversion.

Less efficient recombination has been reported in interspecific crosses due to the genetic divergence between the parental lines belonging to different species (Williams *et al.* 1995; Bernacchi and Tanksley 1997). However, the non-significant difference of either the marker interval sizes between the *Ah* x *Alp* and the *A. lyrata* maps suggests that the recombination between the *A. halleri* and *A. l. petraea* genomes was as efficient in the interspecific hybrid as in the intraspecific cross. Significant differences of linkage group lengths were observed between the *Ah* x *Alp* map and either the *A. l. petraea* map or the *A. l. lyrata* map. Moreover, mean linkage group length was increased in our mapping experiment compared to the *A. lyrata* maps. Since the inverse would be rather expected, we assume that this just indicates the incomplete saturation of the *A. lyrata* genetic linkage maps. We believe that our interspecific map covers most of the *A. halleri* genome, since markers situated near the extremities of *A. thaliana* chromosomes were used in the interspecific cross for map construction. Moreover, the markers located on the extremity of the LG4 (AL4, AlyLG3), LG6 (AL6, AlyLG7) and LG8 (AL8, AlyLG8) lower arms were nearer the *A. thaliana* chromosome extremities in the

interspecific map than in the *A. l. petraea* and the *A. l. lyrata* maps (Kuittinen *et al.* 2004; Yogeeswaran *et al.* 2005).

The genome sizes reported for *A. thaliana* (0.16 pg) and its close relatives (~ 0.26 pg) (Johnstone *et al.* 2005) indicate that one or more deletion events might have accompanied the transition from eight to five chromosomes, characterising the genome of *A. thaliana*. Ideally, the comparative analyses of the *Ah* x *Alp* and the *A. lyrata* maps with the *A. thaliana* genome should take these events into account. In this regard the sequencing project on *A. l. petraea* started in 2005 will be very valuable, since this will provide us an exhaustive knowledge of the genome of the Arabidopsis relatives (http://www.jgi.doe.gov/sequencing/why/CSP2006/AlyrataCrubella.html).

Segregation distortion

At a significance threshold of 0.05, we might expect 5% of all markers to show distorted segregation, by chance; in the BC1 population we greatly exceeded this proportion. A failure to show the expected Mendelian ratios is rather common in interspecific crosses (Zamir and Tadmor 1986; Bernacchi and Tanksley 1997; Jenczewski *et al.* 1997). In a meta-analysis Jenczewski *et al.* (1997) estimated that 28.7% +/- 17.7 of all markers showed segregation distortion in interspecific crosses ($\alpha = 0.05$). In intraspecific crosses segregation distortion has also been reported. However, deviations from Mendelian ratios should occur at lower incidence levels in intraspecific crosses than in interspecific ones (18.4% +/- 11 of all markers at $\alpha = 0.05$) (Jenczewski *et al.* 1997). Segregation bias is believed to occur because of the linkage between molecular markers and reproduction-regulating genes that operate in the pre- or post-zygotic phases of reproduction and whose coordination is disrupted following segregation. It has been suggested that segregation bias should increase with the divergence time between the parental lines. The divergence time between the *A. halleri* and *A. l. petraea* species is estimated to be about half the divergence time between *A. thaliana* and *A. l. petraea* (X. Vekemans, personal communication) which have diverged from each other approximately 5.8 MYA (Koch *et al.* 2001). Thus, the divergence between *A. halleri* and *A. l. petraea* could explain, at least partially, the segregation bias observed in the BC1 population. Segregation distortions can also be

due to significantly different genome sizes of the parental individuals, which involve the generation of abnormal chromosomes after recombination (Jenczewski *et al.* 1997). However, similar genome sizes were reported for *A. halleri* and *A. lyrata* (~ 0.26 pg or 255 Mb) (Dart *et al.* 2004; Johnstone *et al.* 2005). Inbreeding depression is also known to cause segregation distortion (Fishman *et al.* 2001). Due to the cross design used in this study (two different *A. l. petraea* individuals), this is unlikely to have played a major role in the distortion observed in the BC1. The large majority of the distorted markers (92%) showed an excess of homospecific versus heterospecific allelic combinations; such a pattern is more in agreement with a distortion due to outbreeding depression. Nevertheless because the F1 and all the BC1 individuals belong to maternal progenies collected on *A. l. petraea*, the highly directional segregation bias observed in the BC1 could also indicate a negative interaction between the *A. halleri* alleles at the nuclear loci (or at closely linked loci) and the maternally inherited cytoplasmic genotype corresponding to *A. l. petraea* (Fishman *et al.* 2001).

Linkage map construction, and more precisely the estimations of recombination frequencies, can be affected by segregation distortion. A bias in transmission ratios can either result in an increase of the marker distances when pairs of linked markers are distorted in the opposite direction or cause a tight clustering of markers or spurious linkage between markers, when they are distorted in the same direction (Fishman *et al.* 2001; Kuittinen *et al.* 2004). However, because we applied stringent goodness-of–fit thresholds to minimize the effects of segregation distortion on the linkage map construction, and observed macrosynteny between the *Ah* x *Alp* map and the *A. l. petraea* map, as well as between the *Ah* x *Alp* map and *A. thaliana,* we believe that the current map is quite robust.

QTL analysis of Zn tolerance

The QTLs for Zn tolerance explained 42% of the genetic variance. The large part of the genetic variance that still remains unexplained could be due to an underestimation of the number or effects of QTLs. Beavis (1994) conducted a comparative study on the number of QTLs detected for plant height in maize and their effects in several independent studies. The so-called "Beavis effect" predicts that in

experiments using progeny sizes of approximately 100 individuals, fewer QTLs are identified than with larger progeny sizes of about 400. Moreover, estimates of genetic effects were reported to be inflated in experiments using progeny sizes of 100 compared to the ones using progenies of 400 individuals (Beavis 1994; Kearsey and Farquhar 1998; Xu 2003). According to the "Beavis effect" a progeny of intermediate size (~200 individuals), as the one used in this QTL analysis of Zn tolerance, still suffers from a reduction in QTL detection power and an inflation of the estimates of the QTL effects (Beavis 1994; Kearsey and Farquhar 1998; Xu 2003). Such a reduction in power, leading to a failure to detect a number of QTLs in this experiment, could explain the difference observed between broad-sense heritability of Zn tolerance in the BC1 progeny and the variance explained by the QTLs. Segregation distortion is also believed to reduce the power of QTL detection and to affect the estimates of QTL effects, because it reduces the effective size of the progeny by reducing the size of one genotypic class (Bradshaw *et al.* 1998). Segregation distortion was reported for 40% of all markers in the BC1 population. It is therefore possible that some QTLs for Zn tolerance, probably of minor effect, have not been detected. Of the QTLs that have been detected, only the QTL Zntol-3 is located in a distorted region and showed a deficit in heterospecific allelic combinations. It is highly probable that this affected the estimation of the QTL effect since the mean Zn tolerance value was calculated on less heterospecific genotypes than the one calculated on the homospecific genotypes.

Genetic architecture of Zn tolerance in *A. halleri*

We inferred from the QTL analysis that at least three genes are involved in the development of Zn tolerance in the *A. halleri* species, which differs from the previously reported single major gene hypothesized by Macnair *et al.* (1999). In Macnair's study however, Zn tolerance was evaluated at a fixed concentration (250 μM) (Macnair *et al.* 1999), whereas in our study a multiple concentration test was applied to measure tolerance of the BC1 individuals because the latter is assumed more appropriate for assessing quantitative variations in tolerance levels (Schat and ten Bookum, 1992). Moreover, Macnair *et al.* (1999) used the lack of chlorosis as a subjective measure of tolerance rather than root growth. Additionally, the *A. halleri* genotypes used in both studies did not originate from the same metalliferous site. The *A. halleri* individual used

in the QTL analysis was collected from Auby (France), a site with a very high metal contamination of relatively recent date (beginning of the 20th century) resulting from the proximity to a Zn smelter factory (Van Rossum *et al.* 2004). In contrast, the *A. halleri* genotype used in Macnair's study (Macnair *et al.* 1999) originated from a suburb of Langelsheim (Germany); this site was reported to have become contaminated because of medieval metal-mining activities (Weber *et al.* 2004). However, historical and genealogical data suggest that *A. halleri* has been introduced in France from metallicolous sites such as Langelsheim located in Germany (Pauwels *et al.* 2005). Consequently, the different origin of *A. halleri* in both studies is not expected to contribute significantly to the discrepancies observed, even though the existence of specific local adaptations to metal contamination in both *A. halleri* populations might not be excluded.

Pairwise epistatic interactions between the QTLs are observed although at a low significance threshold and should therefore be interpreted with caution. An increase of Zn tolerance caused by the allele of the *A. halleri* genotype was observed at the three QTL positions. Orr (1998) developed a test to infer from the QTL data whether the trait has evolved under neutrality or has rather been subjected to natural selection. QTLs with opposing effects would indicate that the trait has diverged under neutrality, whereas QTLs with effects in the same direction would be expected if the trait has had a continuous history of directional selection (Orr 1998). The positive QTL effects observed for Zn tolerance in our experiment could be the sign of the high levels of directional selection to which Zn tolerance has been subjected in *A. halleri.* Although the low number of QTLs identified precludes the rejection of the hypothesis of neutral divergence (Rieseberg *et al.* 2002), high selective pressures implemented by the elevated heavy metal concentrations of the soil are expected in metallicolous populations. Consequently, these selective pressures should give rise to high natural selection, favoring those mutations that produce an increase in Zn tolerance. Although it is not surprising to observe positive effects for the Zn tolerance QTLs using a metallicolous *A. halleri* individual, we cannot exclude the possibility of identifying QTLs with opposite effects in crosses involving non-metallicolous *A. halleri* individuals.

Co-localisation of known heavy metal homeostasis genes with the QTLs for Zn tolerance

The length of the LOD support intervals associated with the QTLs for Zn tolerance reported in this experiment precludes the direct identification of the underlying genes. In *A. thaliana* for instance, 1 cM has been reported to correspond to an average of 250 kb or approximately 40 genes (Mauricio 2001). However, the correspondence between genetic and physical distances in the close relatives of *A. thaliana* is not known. In this context the sequencing of the *A. l. petraea* genome (http://www.jgi.doe.gov/sequencing/why/CSP2006/AlyrataCrubella.html) will be very useful. On the current map we reported the co-localisation of three genes, *HMA4*, *MTP1-A* and *MTP1-B* with the three QTL regions for Zn tolerance.

HMA4, a member of the family of P-type ATPases co-localised with the QTL Zntol-1. In *A. thaliana*, HMA4 is mainly expressed in the roots and was shown to be involved in the root to shoot transport of Zn and Cd (Mills *et al.* 2003; Hussain *et al.* 2004). Compared to the *A. thaliana* orthologous gene, *HMA4* was shown to be highly overexpressed in the roots of the Zn/Cd-tolerant and hyperaccumulator species *Thlaspi caerulescens,* indicating a possible role in translocation, as well as in the shoots where *HMA4* may be involved in Zn/Cd detoxification (Bernard *et al.* 2004; Papoyan and Kochian 2004). The metal homeostasis genes *MTP1-A* and *MTP1-B* mapped to the QTLs Zntol-2 and Zntol-3 respectively. These genes are homologous to *MTP1* from *A. thaliana*, formerly known as ZAT, a cation diffusion facilitator (CDF) and were clearly shown by functional analysis to interact with zinc homeostasis in *A. halleri* (Dräger *et al.* 2004; Krämer 2005). The co-localisation with the QTLs for Zn tolerance and the differential expression and/or regulation demonstrated for *AhMTP1-A* and *AhMTP1-B* compared to the *AtMTP1* (Dräger *et al.* 2004) in response to Zn, provide strong arguments in favour of adaptive modifications of these specific metal homeostasis genes (or their regulatory regions) in relation with Zn tolerance in *A. halleri.* This may also be the case for *HMA4*, which was described previously in *T. caerulescens* (Bernard *et al.* 2004; Papoyan and Kochian 2004).

In conclusion, our search for QTLs conferring Zn tolerance in *A. halleri* revealed three genomic regions in which three metal homeostasis genes localised. In order to minimize the LOD support intervals associated with the QTL, we are currently increasing the marker density of the *Ah* x *Alp* map and producing second generation backcross progenies. Finally, the *Ah* x *Alp* map constitutes a powerful tool available for the scientific community working on metal homeostasis genes: any gene of interest can be mapped on our material and characterised for its relationships with the QTLs of Zn tolerance in *A. halleri*.

Acknowledgements

We thank Robert Dron, Claire Feutrie and Eric Schmitt for technical advice and support in taking care of the plants, and Maxime Pauwels and Stéphane Fenart for help in phenotyping. We are very grateful to Mark Macnair for providing *A. lyrata* seeds, and to Vincent Castric, Adrian Radu Craciun, Marc Hanikenne, Charles Langley, Michel Lebrun, Stéphanie Loubet, Marie Mirouze, Tom Mitchell-Olds, Ronald Oomen, Outi Savolainen, Christian Schlötterer, Sébastien Thomine and Fabrice Varoquaux for providing primer pairs and/or help in genotyping. We are also very grateful to Steve Barnes, Joel Cuguen, Ute Krämer, Patrick de Laguérie, Henk Schat, Pascal Touzet and Xavier Vekemans for scientific discussions and helpful comments on the manuscript. This research was supported by the Contrat de Plan Etat/Région Nord-Pas de Calais (PRC), the European FEDER (contract no. 79/1769) and the Programme National/ACI-FNS ECCO (contract no. 04 2 9 FNS), in addition to a grant to G.W. from the European Research Training Network Metalhome (HPRN-CT-2002-00243).

4.5 References

Alonso-Blanco, C., El-Assal, S. E., Coupland, G., and Koornneef, M., 1998 Analysis of natural allelic variation at flowering time loci in the Landsberg *erecta* and Cape Verde Islands ecotypes of *Arabidopsis thaliana*. *Genetics* **149:** 749-764.

Alonso-Blanco, C., and Koornneef, M., 2000 Naturally occurring variation in *Arabidopsis*: an underexploited resource for plant genetics. *Trends in Plant Science* **5:** 22-29.

Beavis, W. D., 1994 The power and deceit of QTL experiments: lessons from comparative QTL studies, pp. 250-266 in *Proceedings of the 49th Annual Corn and Sorghum Industry Research Conference*, edited by A. S. Trade, Washington D.C.

Becher, M., Talke, I. N., Krall, L., and Krämer, U., 2004 Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. *Plant Journal* **37:** 251-268.

Bell, C., and Ecker, J., 1994 Assignment of 30 microsatellite loci to the linkage map of *Arabidopsis*. *Genomics* **19:** 137-144.

Bernacchi, D., and Tanksley, S. D., 1997 An interspecific backcross of *Lycopersicon esculentum* x *L. hirsutum*: linkage analysis and a QTL study of sexual compatibility factors and floral traits. *Genetics* **147:** 861-877.

Bernard, C., Roosens, N., Czernic, P., Lebrun, M., and Verbruggen, N., 2004 A novel CPx-ATPase from the cadmium hyperaccumulator *Thlaspi caerulescens*. *FEBS Letters* **569:** 140-148.

Bert, V., Macnair, M. R., de Laguerie, P., Saumitou-Laprade, P., and Petit, D., 2000 Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri* (Brassicaceae). *New Phytologist* **146:** 225-233.

Bert, V., Bonnin, I., Saumitou-Laprade, P., de Laguerie, P., and Petit, D., 2002 Do *Arabidopsis halleri* from nonmetallicolous populations accumulate zinc and cadmium more effectively than those from metallicolous populations ? *New Phytologist* **155:** 47-57.

Boivin, K., Acarkan, A., Mbulu, R. S., Clarenz, O., and Schmidt, R., 2004 The Arabidopsis genome sequence as a tool for genome analysis in Brassicaceae. A

comparison of the Arabidopsis and *Capsella rubella* genomes. *Plant Physiology* **135:** 735-744.

Bradshaw, H. D., Jr., Otto, K. G., Frewen, B. E., McKay, J. K., and Schemske, D. W., 1998 Quantitative Trait Loci affecting differences in floral morphology between two species of monkeyflower (Mimulus). *Genetics* **149:** 367-382.

Clauss, M. J., Cobban, H., and Mitchell-Olds, T., 2002 Cross-species microsatellite markers for elucidating population genetic structure in *Arabidopsis* and *Arabis* (Brassicaeae). *Molecular Ecology* **11:** 591-601.

Clemens, S., 2001 Molecular mechanisms of plant metal tolerance and homeostasis. *Planta* **212:** 475-486.

Dart, S., Kron, P., and Mable, B. K., 2004 Characterizing polyploidy in *Arabidopsis lyrata* using chromosome counts and flow cytometry. *Canadian Journal of Botany* **82:** 185-197.

Doebley, J., Stec, A., and Hubbard, L., 1997 The evolution of apical dominance in maize. *Nature*: 485-488.

Doerge, R. W., 2002 Mapping and analysis of quantitative trait loci in experimental populations. *Nature Reviews Genetics* **3:** 43-52.

Dräger, D. B., Desbrosses-Fonrouge, A.-G., Krach, C., Chardonnens, A. N., Meyer, R. C., Saumitou-Laprade, P., *et al.*, 2004 Two genes encoding *Arabidopsis halleri* MTP1 metal transport proteins co-segregate with zinc tolerance and account for high *MTP1* transcript levels. *Plant Journal* **39:** 425-439.

Erickson, D. L., Fenster, C. B., Stenoien, H. K., and Price, D., 2004 Quantitative trait locus analyses and the study of evolutionary process. *Molecular Ecology* **13:** 2505-2522.

Fishman, L., Kelly, A. J., Morgan, E., and Willis, J. H., 2001 A genetic map in the *Mimulus guttatus* species complex reveals transmission ratio distortion due to heterospecific interactions. *Genetics* **159:** 1701-1716.

Flint, J., and Mott, R., 2001 Finding the molecular basis of quantitive traits: successes and pitfalls. *Nature Reviews Genetics* **2:** 437-445.

Glazier, A. M., Nadeau, J. H., and Aitman, T. J., 2002 Finding genes that underlie complex traits. *Science* **298:** 2345-2349.

Hall, J. L., 2002 Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of Experimental Botany* **53:** 1-11.

Hartley-Whitaker, J., Ainsworth, G., Vooijs, R., Ten Bookum, W., Schat, H., and Meharg, A. A., 2001 Phytochelatins are involved in differential arsenate tolerance in *Holcus lanatus*. *Plant Physiology* **126:** 299-306.

Hussain, D., Haydon, M. J., Wang, Y., Wong, E., Sherson, S. M., Young, J., *et al.*, 2004 P-Type ATPase Heavy Metal Transporters with roles in essential zinc homeostasis in Arabidopsis. *Plant Cell* **16:** 1327-1339.

Jenczewski, E., Gherardi, M., Bonnin, I., Prosperi, J. M., Olivieri, I., and Huguet, T., 1997 Insight on segregation distortions in two intraspecific crosses between annual species of *Medicago* (Leguminosae). *Theoretical and Applied Genetics* **94:** 682-691.

Johnstone, J. S., Pepper, A. E., Hall, A. E., Chen, Z. J., Hodnett, G., Drabek, J., *et al.*, 2005 Evolution of genome size in Brassicaceae. *Annals of Botany* **95:** 229-235.

Kearsey, M. J., and Farquhar, A. G., 1998 QTL analysis in plants; where are we now? *Heredity* **80:** 137-142.

Koch, M., Haubold, B., and Mitchell-Olds, T., 2001 Molecular systematics of the Brassicaceae: evidence from coding plastidic matK and nuclear Chs sequences. *American Journal of Botany* **88:** 534-544.

Koch, M. A., and Kiefer, M., 2005 Genome evolution among cruciferous plants: a lecture from the comparison of the genetic maps of three diploid species--*Capsella rubella*, *Arabidopsis lyrata* subsp. *petraea*, and *A. thaliana*. *American Journal of Botany* **92:** 761-767.

Koornneef, M., Alonso-Blanco, C., and Vreugdenhil, D., 2004 Naturally occurring genetic variation in *Arabidopsis thaliana*. *Annual Review of Plant Biology* **55:** 141-172.

Kosambi, D. D., 1944 The estimation of map distances from recombination values. *Annual Eugenetics* **12:** 172-175.

Krämer, U., Cotter-Howells, J. D., Charnock, J. M., Baker, A. J. M., and Smith, A. C., 1996 Free histidine as a metal chelator in plants that accumulate nickel. *Nature* **379:** 635-638.

Krämer, U., 2005 MTP1 mops up excess zinc in *Arabidopsis* cells. *Trends in Plant Science* **10:** 313-315.

Kuittinen, H., Aguadé, M., Charlesworth, D., De Haan, A., Lauga, B., Mitchell-Olds, T., *et al.*, 2002 Primers for 22 candidate genes for ecological adaptations in Brassicaceae. *Molecular Ecology Notes* **2:** 258-262.

Kuittinen, H., de Haan, A. A., Vogl, C., Oikarinen, S., Leppala, J., Koch, M., *et al.*, 2004 Comparing the linkage maps of the close relatives *Arabidopsis lyrata* and *A. thaliana*. *Genetics* **168:** 1575-1584.

Mackay, T. F., 2001 The genetic architecture of quantitative traits. *Annual Review of Genetics* **35:** 303-339.

Macnair, M. R., 1993 Tansley review No. 49: The genetics of metal tolerance in vascular plants. *New Phytologist* **124:** 541-559.

Macnair, M. R., Bert, V., Huitson, S. B., Saumitou-Laprade, P., and Petit, D., 1999 Zinc tolerance and hyperaccumulation are genetically independent characters. *Proceedings of the Royal Society of London, Series B: Biological Sciences* **266:** 2175-2179.

Mauricio, R., 2001 Mapping Quantitative Trait Loci in plants: uses and caveats for evolutionary biology. *Nature Reviews Genetics* **2:** 370-380.

Mills, R., Krijger, G., Baccarini, P., Hall, J. L., and Williams, L., 2003 Functional expression of AtHMA4, a P_{1B}-type ATPase of the Zn/Co/Cd/Pb subclass. *Plant Journal* **35:** 164-176.

Mitchell-Olds, T., 2001 *Arabidopsis thaliana* and its wild relatives: a model system for ecology and evolution. *Trends in Ecology and Evolution* **16:** 693-700.

Oetting, W. S., Lee, H. K., Flanders, D. J., Wiesner, G. L., Sellers, T. A., and King, R. A., 1995 Linkage analysis with multiplexed short tandem repeat polymorphism using infrared fluorescence and M13 tailed primers. *Genomics* **30:** 450-458.

Olsen, K. M., and Purugganan, M. D., 2002 Plant population genomics, linkage disequilibrium mapping, and the genetics of adaptation *Plant Adaptation: Molecular Genetics and Ecology.*, edited by J. W. Q.C.B. Cronk, R. H. Ree and I.E.P. Taylor, Vancouver, British Columbia, Canada.

Orr, H. A., 1998 Testing natural selection vs. genetic drift in phenotypic evolution using Quantitative Trait Locus data. *Genetics* **149:** 2099-2104.

Papoyan, A., and Kochian, L. V., 2004 Identification of *Thlaspi caerulescens* genes that may be involved in heavy metal hyperaccumulation and tolerance. Characterization of a novel heavy metal transporting ATPase. *Plant Physiology* **136:** 3814-3823.

Pauwels, M., Saumitou-Laprade, P., Holl, A., Petit, D., and Bonnin, I., 2005 Multiple origin of metallicolous populations of the pseudometallophyte *Arabidopsis halleri* (Brassicaceae) in central Europe: the cpDNA testimony. *Molecular Ecology* **14:** 4403-4414.

Rieseberg, L. H., Widmer, A., Arntz, A. M., and Burke, J. M., 2002 Directional selection is primary cause of phenotypic diversification. *Proceedings of the National Academy of Sciences of the USA* **99:** 12242-12245.

SAS, 1999 Statistical Analysis Systems, pp. SAS Institute Inc., Cary, NC.

Saumitou-Laprade, P., Piquot, Y., Raspé, O., Bernard, J., and Vrieling, K., 1999 Plant DNA fingerprinting and profiling, pp. 17-38 in *DNA profiling and DNA fingerprinting*, edited by J. T. Epplen and T. Lubjuhn. Birkhauser, Basel.

Schat, H., and Ten Bookum, W. M., 1992 Genetic control of copper tolerance in *Silene vulgaris*. *Heredity* **68:** 219-229.

Schat, H., Kuiper, E., Ten Bookum, W. M., and Vooijs, R., 1993 A general model for the genetic control of copper tolerance in *Silene vulgaris*: evidence from crosses between plants from different tolerant populations. *Heredity* **70:** 142-147.

Schat, H., Llugany, M., Vooijs, R., Hartley-Whitaker, J., and Bleeker, P. M., 2002 The role of phytochelatins in constitutive and adaptive heavy metal tolerances in hyperaccumulator and non-hyperaccumulator metallophytes. *Journal of Experimental Botany* **53:** 2381-2392.

Smith, S. E., and Macnair, M. R., 1998 Hypostatic modifiers cause variation in degree of copper tolerance in *Mimulus guttatus*. *Heredity* **80:** 760-768.

Tanksley, S. D., 1993 Mapping polygenes. *Annual Review of Genetics* **27:** 205-233.

Ungerer, M. C., Halldorsdottir, S. S., Modliszewski, J. L., Mackay, T. F., and Purugganan, M. D., 2002 Quantitative trait loci for inflorescence development in *Arabidopsis thaliana*. *Genetics* **160:** 1133-1151.

Van Ooijen, J. W., and Voorrips, R. E., 2001 Joinmap 3.0, Software for the calculation of genetic linkage maps, Plant Research International, Wageningen, the Netherlands.

Van Ooijen, J. W., Boer, M. P., Jansen, R. C., and Maliepaard, C., 2002 MapQTL 4.0, Software for the calculation of QTL positions on genetic maps, Plant Research International, Wageningen, the Netherlands.

Van Rossum, F., Bonnin, I., Fenart, S., Pauwels, M., Petit, D., and Saumitou-Laprade, P., 2004 Spatial genetic structure within a metallicolous population of *Arabidopsis halleri*, a clonal, self-incompatible and heavy-metal-tolerant species. *Molecular Ecology* **13:** 2959-2967.

Voorrips, R. E., 2002 Mapchart: Software for the graphical presentation of linkage maps and QTLs. *Heredity* **93:** 77-78.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., and van de Lee, T., 1995 AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23:** 4407-4414.

Weber, M., Harada, E., Vess, C., Roepenack-Lahaye, E., and Clemens, S., 2004 Comparative microarray analysis of *Arabidopsis thaliana* and *Arabidopsis halleri* roots identifies nicotianamine synthase, a ZIP transporter and other genes as potential metal hyperaccumulation factors. *Plant Journal* **37:** 269-281.

Weinig, C., Stinchcombe, J. R., and Schmitt, J., 2003 QTL architecture of resistance and tolerance traits in *Arabidopsis thaliana* in natural environments. *Molecular Ecology* **12:** 1153-1163.

Williams, C. G., Goodman, M. M., and Stuber, C. W., 1995 Comparative recombination distances among *Zea mays* L. inbreds, wide crosses and interspecific hybrids. *Genetics* **141:** 1573-1581.

Xu, S., 2003 Theoretical basis of the Beavis effect. *Genetics* **165:** 2259-2268.

Yano, M., 2001 Genetic and molecular dissection of naturally occurring variation. *Current Opinion in Plant Biology* **4:** 130-135.

Yogeeswaran, K., Frary, A., York, T. L., Amenta, A., Lesser, A. H., Nasrallah, J. B., *et al.*, 2005 Comparative genome analyses of *Arabidopsis* spp.: Inferring chromosomal rearrangement events in the evolutionary history of *A. thaliana*. *Genome Research* **15:** 505-515.

Zamir, D., and Tadmor, Y., 1986 Unequal segregation of nuclear genes in plants. *Botanical Gazette* **147:** 355-358.

5 QUANTITATIVE TRAIT LOCI MAPPING OF CADMIUM TOLERANCE IN THE METALLOPHYTE *ARABIDOPSIS HALLERI*

5.1 Introduction

Contrary to zinc (Zn), cadmium (Cd) is a nonessential element, since to date no biological function has been identified for this metal. Similarly to most other heavy metals high, and consequently toxic, Cd concentrations are released in the environment predominantly by the industrial activity (Sanito di Toppi and Gabbrielli 1999). Whereas unpolluted soils are considered to contain generally less than 2 $\mu g\ g^{-1}$ available Cd, in mine soils Cd concentrations can easily reach 60 $\mu g\ g^{-1}$ (Bert *et al.* 2002). Moreover, industrial polluted soils generally possess elevated concentrations of a combination of heavy metals rather than of just one single element. This is particularly true for Cd-polluted sites because Cd is mostly produced as a by-product of lead (Pb) and Zn mineralization (Sanito di Toppi and Gabbrielli 1999). In response to the hostile environmental conditions implied by the presence of high metal concentrations in the soil some plants species developed an efficient adaptation enabling them to thrive on heavy metal polluted sites (Macnair *et al.* 2000). *A. halleri* for instance has the ability to withstand high Zn and Cd concentrations, which allows *A. halleri* plants to colonise calamine soils containing high Pb, Zn and Cd concentrations (Bert *et al.* 2000).

Heavy metal tolerance, established in the literature as one of the best examples of adaptation (Antonovics *et al.* 1971), is a complex phenomenon and little is yet known about the precise genetic architecture and physiological mechanisms underlying this trait. Segregation analyses of tolerance to various metals in various species indicated the

existence of a small number of major genes, with a predominant additive effect (Schat and Ten Bookum 1992; Macnair 1993). With regard to the identification and characterisation of genes potentially involved in heavy metal tolerance most progress has been made in the model species *A. thaliana* (Thomine *et al.* 2000; Desbrosses-Fonrouge *et al.* 2005; Verret *et al.* 2005) and direct evidence for the implication of a particular gene in heavy metal tolerance in metallophyte species could be reported only in few cases (Hartley-Whitaker *et al.* 2001; Dräger *et al.* 2004). Recently, it was demonstrated that the Quantitative Trait Loci (QTL) mapping method could be successfully used for addressing the genetic, and eventually physiological bases of heavy metal tolerance (Willems *et al.* submitted) or hyperaccumulation (Assunçao *et al.* 2005) in metallophyte species. Through a QTL analysis conducted on Zn tolerance in the *A. halleri* species three genomic regions were identified, explaining 42% of the genetic variance. Moreover, through the use of sequence-based markers anchored in the *A. thaliana* genome and the mapping of seven genes with a function in metal homeostasis, potential candidate genes could be proposed. Based on the co-localisation of the metal transporters MTP1-A, MTP1-B and HMA4 with the QTLs for Zn tolerance, a function for these proteins in Zn tolerance can be expected.

In this study we applied a Quantitative Trait Loci (QTL) mapping approach to study the genetic architecture of Cd tolerance in *A. halleri*. In *A. halleri* two or three major genes with additive effect were hypothesised to govern Cd tolerance upon the analysis of its segregation in an *A. h. halleri* x *A. l. petraea* backcross progeny (Bert *et al.* 2003). Metal transporters belonging to the ZIP family (Guerinot 2000), the NRAMP family (Thomine *et al.* 2000) and the HMAs (Mills *et al.* 2003; Bernard *et al.* 2004; Papoyan and Kochian 2004) have been reported to be involved in Cd homeostasis, even though for none of these transporters circumstantial evidence in favour of a role in Cd tolerance has been provided yet. Chelation of Cd by phytochelatins (PCs) was initially believed to contribute importantly to adaptive Cd tolerance, based on the findings that the synthesis of PCs was induced by the concentration of Cd in the cell as well as on the analysis of Cd-hypersensitive *A. thaliana* mutants which were PC-deficient (Cobbett 2000; Cobbett and Goldsbrough 2002). However, a function for PCs in Cd tolerance in *S. vulgaris* at least could not be supported. The synthesis of PCs was consistently higher

in Cd-sensitive *S. vulgaris* populations when compared to Cd-tolerant populations. In addition, no increased sensitivity was observed in the Cd-tolerant populations upon the inhibition of PC synthesis (de Knecht *et al.* 1995; Schat *et al.* 2002). All together the genetic architecture of Cd tolerance as well as the precise mechanisms underlying this process remain largely unresolved. Similarly to Zn tolerance the QTL mapping approach might therefore be useful for elucidating the mechanisms involved in Cd tolerance in *A. halleri*. The mapping population that was used for identifying the QTLs conferring Zn tolerance, i.e. an *A. halleri* x *A. l. petraea* backcross progeny showed to segregate for Cd tolerance too. The use of an identical mapping population and thus, an identical genetic map enabled us to analyse the existence of common genetic mechanisms in both Zn and Cd tolerance.

5.2 Materials and methods

Crossing scheme

The crossing scheme applied in the QTL analysis of Cd tolerance is identical to the one used in the QTL analysis of Zn tolerance (Willems *et al.* submitted). However, only a subset of the first generation backcross (BC1) progeny (79 individuals) obtained on the cross between the Zn- and Cd-tolerant *A. h. halleri* individual (pollen donor, Auby, France) (henceforth called *A. halleri*) and the non-tolerant *A. l. petraea* individual (pollen recipient, Unhost, Czech Republic) (henceforth called *A. l. petraea* 1) was used in the QTL mapping of Cd tolerance.

Evaluation of Cd tolerance

The evaluation of Cd tolerance of the four parental genotypes (*A. halleri*, *A. l. petraea* 1, *A. l. petraea* 2 and F1) and 79 BC1 individuals was performed by V. Bert, as described in Bert *et al.* (2003). Cd tolerance was measured on three clonal replicates of each individual in a sequential exposure test in hydroponic solution (Schat and Ten Bookum 1992). The nutrient solution was composed of 0.5 mM $Ca(NO_3)$, 0.2 mM $MgSO_4$, 0.5 mM KNO_3, 0.1 mM K_2HPO_4, 0.2 µM $CuSO_4$, 2 µM $MnCl_2$, 10 µM H_3BO_3, 0.1 µM MoO_3, 10 µM FeEDDHA and 10 to 250 µM Cd added as $CdCl_2$. The pH of the

solution was set at 6.5. The plants were randomly disposed in trays and randomisation of the trays in the growth chamber was performed weekly. The hydroponic solution was renewed once a week while increasing the Cd concentration (10, 25, 50, 75, 100, 150 and 250 μM). To easily observe new root growth the roots were blackened with activated charcoal. At the end of each week the root growth of each individual was evaluated as well as its total biomass after gently drying the roots of the plant with tissue paper. Tolerance was determined as the lowest concentration (the EC100) at which no new root growth and no increase in biomass was observed (Bert *et al.* 2003).

QTL mapping of Cd tolerance

The genotype effect was estimated by a one-way analysis of variance using the GLM procedure in SAS (SAS 2001). The broad-sense heritability of Cd tolerance in the BC1 was calculated using the mean square values (MS) of ANOVA analysis ($h^2 = MS_{genot} / (MS_{genot} + MS_{error})$. Type III sums of squares were used because the data set was unbalanced due to the unequal number of replicates of each BC1 individual.

The QTL analysis was performed using the MapQTL 4.0 program (Van Ooijen *et al.* 2002). QTLs of Cd tolerance were identified using $EC100_{mean}$ values of the BC1 genotypes, i.e. the arithmetic mean on the EC100 values of the clones. QTLs were positioned on the *A. halleri* x *A. l. petraea* (henceforth called the *Ah* x *Alp* map) genetic map presented in Willems *et al.* (submitted). The significance threshold of the LOD score for QTL detection was obtained by performing a permutation test on the quantitative data as implemented in MapQTL. The threshold was set at a LOD score of 2.4 corresponding to an error level $\alpha = 0.05$. A first QTL analysis was performed using interval mapping (IM). In IM LOD scores were calculated every centiMorgan (cM) within the intervals along the linkage groups. The markers for which the LOD score exceeded the significance threshold were included in an automatic cofactor selection analysis and the selected markers were then used as cofactors in MQM analysis. In MQM mapping a one-dimensional search over the genome is performed as in IM, while simultaneously fitting the selected cofactors that take over the role of the other QTLs. In the QTL analysis of Cd tolerance MQM mapping was performed twice while adjusting the set of cofactors to obtain the best possible set of QTLs, i.e. showing maximal LOD

scores. One- and two-LOD support intervals were obtained using Mapchart 2.1 (Voorrips 2002). The estimated additive genetic effect (*a*) and the percentage of variance explained by each QTL (R^2) were calculated in IM. Additionally, we tested for significant interactions between QTLs using the GLM procedure in SAS (SAS 2001). The markers closest to or at the QTL position were considered as random factors in this analysis.

5.3 Results

Evaluation of Cd tolerance

The ANOVA analysis performed on the EC100 values[6] revealed a highly significant genotype effect ($P < 0.0001$); a high broad-sense heritability was obtained for Cd tolerance ($h^2 = 0.89$). Large variation for Cd tolerance was observed in the BC1 progeny; individuals with tolerance levels identical to both parental genotypes were present within the BC1 progeny (Figure 4.1).

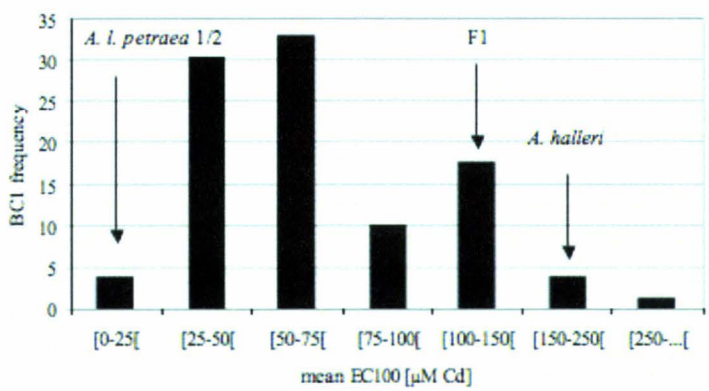


Figure 5.1 Frequency distribution of Cd tolerance in a BC1 progeny derived from an *A. halleri* x *A. l. petraea* cross.

Cd tolerance of the 79 BC1 individuals and the four parental genotypes was evaluated by a sequential exposure test in hydroponic culture. The Cd tolerance levels of the parental genotypes (Bert *et al.* 2003) are indicated by arrows above the appropriate tolerance class.

[6] See Annex TABLE 2 for EC100 values

QTL mapping of Cd tolerance

Through IM and MQM analysis three QTLs Cdtol-1, Cdtol-2 and Cdtol-3 were detected on the linkage groups LG3, LG4 and LG6 of the *Ah* x *Alp* linkage map (Figure 5.2).

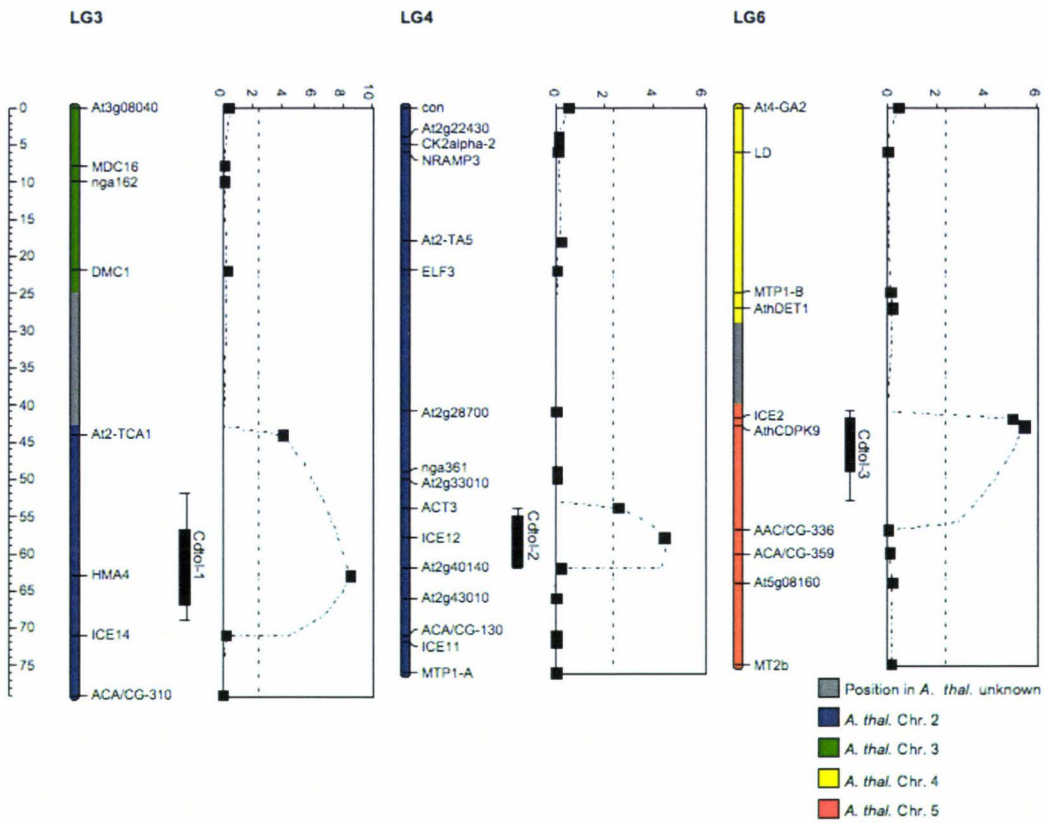


Figure 5.2 LOD score profiles and LOD support intervals for the QTLs conferring Cd tolerance in the BC1 progeny.

The LOD score profiles are given at the right of the linkage groups LG3, LG4 and LG6. On the horizontal axis the LOD score is plotted; on the vertical axis the map position (cM) is plotted. The LOD score of each marker, indicating the position of the QTL, is represented by a black square on the profile. The dashed line indicates the LOD score threshold of 2.4. The most likely position of a QTL corresponds to the map position where the LOD score reaches its maximum, while exceeding the LOD score threshold. The confidence intervals or LOD support intervals associated with the QTLs correspond to the interval over which the LOD score is within one or two log-units of its maximum value. Filled black bars indicate 1-LOD (10-fold) support intervals and whiskers (lines extending beyond bars) indicate 2-LOD (100-fold) support intervals. The ruler at the left of the figure indicates the map position (cM). The homology with the *A. thaliana* chromosomes is indicated by the colours.

The QTLs Cdtol-1, Cdtol-2 and Cdtol-3 explained respectively 38.3%, 23% and 19.8% of the total phenotypic variance (Table 5.1). All together they accounted for 81.1% of the phenotypic variance or 91% of the genetic variance of Cd tolerance in the BC1 progeny. The one-LOD support intervals associated with the QTLs varied in length from 9 cM for Cdtol-1, to 4 cM for Cdtol-2 and to 8 cM for Cdtol-3. The two-LOD support intervals corresponded to 16 cM, 7 cM and 13 cM for the QTLs Cdtol-1, Cdtol-2 and Cdtol-3 respectively. At all three QTLs the *A. halleri* allele increased Cd tolerance. The increase in Cd tolerance due to the presence of the *A. halleri* allele however, was greater for the QTL Cdtol-1 than for the other QTLs (Table 5.1). One significant epistatic interaction was revealed between the QTLs Cdtol-1 and Cdtol-2, although at a low significance threshold (P = 0.0106). The *HMA4* marker co-localised with Cdtol-1, whereas the markers *ICE12* and *ACT3* and the markers *ICE2* and *AthCDPK9* co-localised with the QTLs Cdtol-2 and Cdtol-3 respectively.

Table 5.1 QTLs for Cd tolerance in *A. halleri* using an *A. halleri* x *A. l. petraea* backcross progeny

QTL[a]	LG-Marker[b]	Position[c]	LOD score[d]	R^{2e}	a[f]
Cdtol-1	LG3-HMA4	63.2	8.52	38.3	-51.0194
Cdtol-2	LG4-ICE12	57.6	4.39	23	-39.4468
Cdtol-3	LG6-AthCDPK9	42.6	5.53	19.8	-38.2731

[a]QTLs are named by the trait and ordered from 1 to 3.
[b]linkage group on which QTLs were mapped and marker at QTL position or closest to QTL position.
[c]position on the linkage group of the marker at or closest to the QTL (in cM).
[d]LOD score of the QTL.
[e] % of explained variance of the QTL.
[f]additive effects of the QTL, corresponding to the differences between the means of the two genotypic groups in BC1 progeny (negative values imply that the *A. halleri* allele increases Zn tolerance compared to the *A. l. petraea* allele).

Co-localisation of the QTLs for Cd and Zn tolerance

Since the QTL analyses of both Zn (Willems *et al.* submitted) and Cd tolerance were performed on the same mapping population, we are able to investigate the co-localisation of QTLs identified for both traits. However, the QTL analysis of Cd

tolerance was performed on only a subset of the BC1 individuals used for the QTL analysis of Zn tolerance.

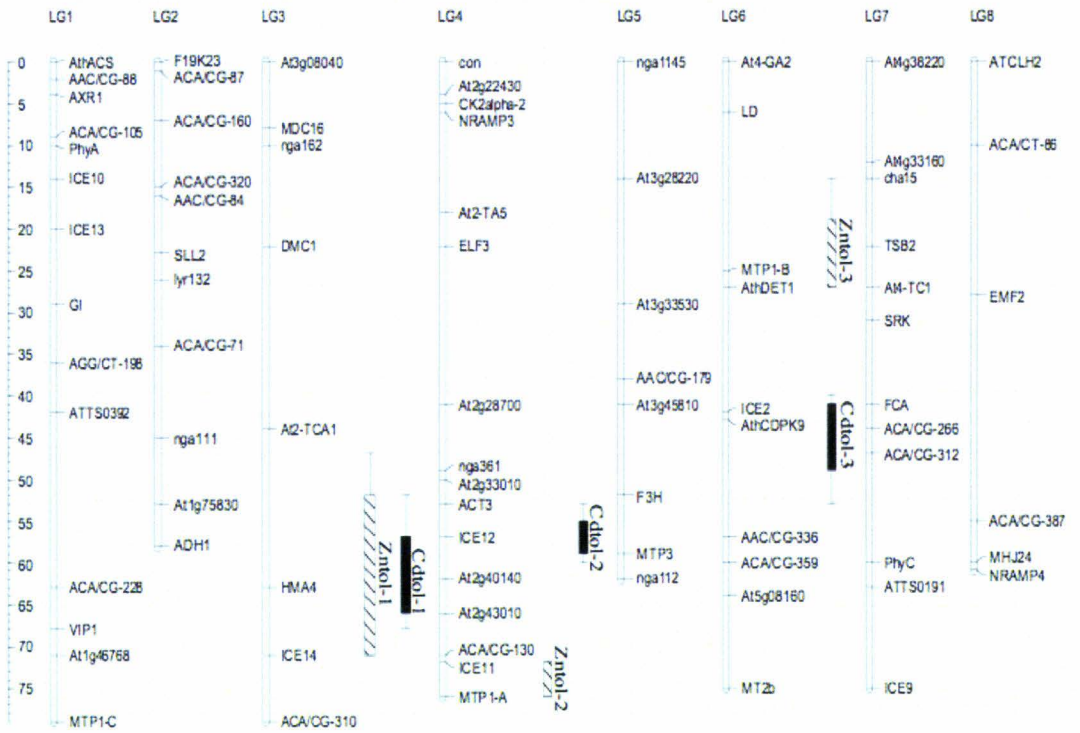


Figure 5.3 Co-localisation of the QTLs for Cd and Zn tolerance in the *A. halleri* x *A. l. petraea* BC1 progeny.

QTLs for Cd and Zn tolerance (Cdtol-1, Cdtol-2 and Cdtol-3; Zntol-1, Zntol-2 and Zntol-3) (Willems *et al.* submitted) are indicated at the right of the linkage groups LG3, LG4 and LG6. The position of a QTL is shown as the interval over which the LOD score is within 1 or 2 log-units of its maximum value, i.e. at the most likely position of the QTL. Bars (filled black bars and dashed bars for Cd and Zn tolerance respectively) indicate 1-LOD (1 log-unit or 10-fold) support intervals; whiskers (lines extending beyond bars) indicate 2-LOD (2 log-units or 100-fold) support intervals. The genetic map, with the positions of the markers indicated by the ruler (cM) is from Willems *et al.* (submitted).

Tolerance to Cd and Zn is governed by at least one common genomic region given the co-localisation of the QTLs Cdtol-1 and Zntol-1 and their associated LOD support intervals. In addition, in both cases the maximum LOD score or the most probable position of the QTL corresponded to the marker *HMA4*. The two other QTLs for Cd tolerance Cdtol-2 and Cdtol-3 were detected on the same linkage groups as the

QTLs Zntol-2 and Zntol-3. Although identified in close proximity of each other, their LOD support intervals did not overlap (Figure 5.3).

5.4 Discussion

QTL analysis of Cd tolerance

The three QTLs detected for Cd tolerance explained almost all of the genetic variance (91%) observed for this trait in the BC1 progeny. Thus, we might consider to have detected all QTLs involved in Cd tolerance. However, some prudence should be adopted in the interpretation of these results. Firstly, we used a relatively small number of BC1 individuals (79) to perform this QTL analysis and as stated by the so-called "Beavis effect", the use of progeny sizes of approximately 100 individuals in QTL experiments results in an underestimation of the number of QTLs as well as in an overestimation of the QTL effects (Beavis 1994). Secondly, the BC1 population used in this study revealed a significant segregation bias. Segregation distortion can affect the estimation of the QTL effects through the reduction of the number of genotypes used for the calculation of the QTL effect (Bradshaw *et al.* 1998). Regarding the QTL regions conferring Cd tolerance the markers *ICE2* and *AthCDPK9* revealed to be distorted. The marker locus *AthCDPK9* was moreover situated at the most likely position of the QTL Cdtol-3. The smaller number of heterospecific (*A. halleri*/*A. l. petraea* 2) genotypes when compared to the homospecific (*A. l. petraea* 1/*A. l. petraea* 2) genotypes at this marker might have affected the estimation of the effects associated with the QTL Cdtol-3.

Genetic architecture of Cd tolerance

The genetic architecture of Cd tolerance was addressed previously by Bert *et al.* (2003) through the analysis of the segregation of Cd tolerance in 66 of the 79 BC1 individuals used in this QTL experiment. The segregation ratios showed to be significantly different from a 1:1 ratio expected for a character controlled by one major gene, while the hypothesis of Cd tolerance controlled by two or three major genes with additive effect could not be rejected (Bert *et al.* 2003). Through the QTL analysis we

identified at least three QTLs governing Cd tolerance in the *A. halleri* x *A. l. petraea* BC1 progeny. At this moment however, we cannot exclude the possibility that several linked genes underlie the QTL regions, which would then increase the total number of genes governing Cd tolerance in *A. halleri*. In addition, the QTL analysis suggested the predominant role of the QTL Cdtol-1 in the genetic control of Cd tolerance in the BC1 progeny. This QTL explained up to 42% of the genetic variance observed for Cd tolerance in the mapping population. Contrary to the classical segregation analyses through which the genetic determinism of heavy metal tolerance has been usually addressed, the QTL analysis provided evidence for the implication of one major QTL and two QTLs of minor effect in Cd tolerance in *A. halleri*. At all three QTLs the *A. halleri* allele increased Cd tolerance, as was previously reported for the QTLs conferring Zn tolerance. The *A. halleri* individual used in this study originated from an industrial polluted site characterised by elevated concentrations of Zn, Cd and Pb. The high selective pressures implemented by the presence of toxic Cd concentrations most probably involved the natural selection of genes increasing Cd tolerance which should give rise to the positive effect on Cd tolerance of the *A. halleri* alleles (Schat and Vooijs 1997). Although this explanation is plausible for metallicolous *A. halleri* individuals, it should be verified for *A. halleri* individuals growing on unpolluted sites. An extensive survey of Cd tolerance in metallicolous and non-metallicolous *A. halleri* populations has not been performed yet, even though the constitutive presence of Cd tolerance in the *A. halleri* species might be expected considering the observation of Cd accumulation in several metallicolous and non-metallicolous *A. halleri* populations (Bert *et al.* 2002). In the latter however, the absence of high selective pressures for Cd tolerance could result in the existence of QTLs with opposite effects.

Identification of potential candidate genes for Cd tolerance

Among the three QTLs Cdtol-1 co-localised with one of the seven metal homeostasis genes, more precisely *HMA4*, that were positioned on the *Ah* x *Alp* map. The metal homeostasis gene *HMA4* belongs to the transporter family of the P-type ATPases (Baxter *et al.* 2003). In *A. thaliana HMA4* was extensively studied with regard to its role in Zn and Cd homeostasis (Hussain *et al.* 2004; Mills *et al.* 2005; Verret *et al.* 2005). Considering the expression of *HMA4* in the vascular bundle of both roots and

shoots, this gene is believed to be involved in xylem loading and unloading of Zn and Cd as well as in the remobilisation of those metals from the shoots to the roots through the phloem (Hussain *et al.* 2004; Verret *et al.* 2004). In the Zn- and Cd-tolerant and hyperaccumulator species *Thlaspi caerulescens*, *HMA4* was constitutively higher expressed in the roots than the *A. thaliana* ortholog (Bernard *et al.* 2004; Papoyan and Kochian 2004). A role of *TcHMA4* in the translocation of Cd from the roots to the shoots was suggested, whereas its expression in the shoots might indicate its implication in Zn/Cd detoxification (Bernard *et al.* 2004; Papoyan and Kochian 2004). In *A. halleri* the gene encoding HMA4 was recently investigated (Courbot *et al.* submitted). Expression analysis of Cd-tolerant and Cd-sensitive individuals of the BC1 progeny indicated a high expression of *AhHMA4* in the roots and the shoots of the tolerant individuals, whereas no signal was observed in the Cd-sensitive genotypes. A high expression of *AhHMA4* was also reported for the F1 and the *A. halleri* parental genotype, contrary to the Cd-sensitive *A. l. petraea* 1 parental line. In all individuals *AhHMA4* was predominantly expressed in the roots. The highest expression however, was reported for the *A. halleri* individual. Moreover, *AhHMA4* seemed to be differentially regulated in the Cd-tolerant genotypes. Whereas no induction of the expression level of *AhHMA4* was observed in *A. halleri* upon treatment with Cd, a clear induction was observed in the F1 and to a lesser extent in the Cd-tolerant BC1 individuals under high (100 µM) and low (10 µM) Cd treatment for the roots and upon exposure to high Cd (100 µM) concentrations in the shoots. The localisation of AhHMA4 in the plasma membrane and its role in the transport of Cd and Zn were consistent with a predicted function of HMA4 in maintaining low cellular Cd and Zn concentrations (Courbot *et al.* submitted).

For the QTLs Cdtol-2 and Cdtol-3 no valuable candidate gene could be suggested, since none of the metal homeostasis genes positioned on the *Ah* x *Alp* map co-localised with the QTL regions. In addition the considerable length of the LOD support intervals (7 and 13 cM for the two-LOD support intervals of Cdtol-2 and Cdtol-3 respectively) precluded the identification of potential candidate genes in the *A. thaliana* genome sequence. Based on the estimation in *A. thaliana* of approximately 40

genes each cM, up to 280 and 520 genes could be located within the QTL regions Cdtol-2 and Cdtol-3 in *A. thaliana* at least.

Co-localisation of the QTLs for Cd and Zn tolerance in *A. halleri*

Because only one QTL region was common to both Zn and Cd tolerance, these traits might be considered under a partially independent genetic control, as previously hypothesised by Bert *et al.* (2003). Moreover, these results are consistent to a large extent with the assumption that functional tolerances, i.e. tolerances to metals present at toxic concentrations in the soil should have evolved through the acquisition of specific genetic mechanisms, whereas non-functional tolerances, i.e. tolerances to metals that are not present at toxic concentrations, should rather be the result of a by-product of an existing functional tolerance (Schat and Vooijs 1997). Since different progeny sizes were used in the QTL analyses of Zn and Cd tolerance, we might however expect that the close proximity of the QTLs Cdtol-2/Zntol-2 and Cdtol-3/Zntol-3 masks the actual overlapping position of these QTLs. To verify this we performed the map construction on the 79 BC1 individuals used in the QTL analysis of Cd tolerance. A genetic linkage map identical in marker order than the one constructed on the complete set of BC1 individuals (199 individuals), but showing slightly different marker distances was obtained. The QTLs for Cd tolerance also localised at the same positions on both linkage maps. For both QTLs Cdtol-1 and Zntol-1 the metal homeostasis gene *HMA4* was situated at the most likely QTL position. This QTL was also the major QTL conferring Cd tolerance. Regarding Zn tolerance the QTL Zntol-1 explained also the greatest part of the phenotypic variance (12.2 %) observed for this trait in the BC1 progeny. However, the part of variance explained by the QTL Zntol-1 was only slightly higher than the one explained by the QTL Zntol-2 (11.2%). Nevertheless, because the metalliferous sites on which metallicolous *A. halleri* populations grow are characterised by high levels of Zn as well as Cd, the contribution of *HMA4* in both Zn and Cd tolerance is interesting. This might for instance suggest that *HMA4* enabled *A. halleri* individuals to colonise calamine soils and that this gene played a major role in the acquisition of Zn and Cd tolerance in *A. halleri*. However a fine mapping analysis of the QTLs for Zn and Cd tolerance has to be performed to confirm the implication of *HMA4*

in Cd and Zn tolerance. To date, functional studies at least provided evidence for a role of HMA4 in both Zn and Cd homeostasis.

5.5 References

Antonovics, J., Bradshaw, A. D., and Turner, R. G., 1971 Heavy metal tolerance in plants. *Advances in Ecological Research* **7:** 1-85.

Assunçao, A. G. L., Pieper, B., Vromans, J., Lindhout, P., Aarts, M. G. M., and Schat, H., 2005 Construction of a genetic linkage map of *Thlaspi caerulescens* and quantitative trait loci analysis of zinc accumulation. *New Phytologist* **0:** ???-???

Baxter, I., Tchieu, J., Sussman, M. R., Boutry, M., Palmgren, M. G., Gribskov, M., *et al.*, 2003 Genomic comparison of P-type ATPase ion pumps in Arabidopsis and rice. *Plant Physiology* **132:** 618-628.

Beavis, W. D., 1994 The power and deceit of QTL experiments: lessons from comparative QTL studies, pp. 250-266 in *Proceedings of the 49th Annual Corn and Sorghum Industry Research Conference*, edited by A. S. Trade, Washington D.C.

Bernard, C., Roosens, N., Czernic, P., Lebrun, M., and Verbruggen, N., 2004 A novel CPx-ATPase from the cadmium hyperaccumulator *Thlaspi caerulescens*. *FEBS Letters* **569:** 140-148.

Bert, V., Macnair, M. R., de Laguerie, P., Saumitou-Laprade, P., and Petit, D., 2000 Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri* (Brassicaceae). *New Phytologist* **146:** 225-233.

Bert, V., Bonnin, I., Saumitou-Laprade, P., de Laguerie, P., and Petit, D., 2002 Do *Arabidopsis halleri* from nonmetallicolous populations accumulate zinc and cadmium more effectively than those from metallicolous populations? *New Phytologist* **155:** 47-57.

Bert, V., Meerts, P., Saumitou-Laprade, P., Salis, P., Gruber, W., and Verbruggen, N., 2003 Genetic basis of Cd tolerance and hyperaccumulation in *Arabidopsis halleri*. *Plant and Soil* **249:** 9-18.

Bradshaw, H. D., Jr., Otto, K. G., Frewen, B. E., McKay, J. K., and Schemske, D. W., 1998 Quantitative Trait Loci affecting differences in floral morphology between two species of monkeyflower (Mimulus). *Genetics* **149:** 367-382.

Cobbett, C., and Goldsbrough, P., 2002 Phytochelatines and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annual Review of Plant Biology* **53:** 159-182.

Cobbett, C. S., 2000 Phytochelatin biosynthesis and function in heavy-metal detoxification. *Current Opinion in Plant Biology* **3:** 211-216.

Courbot, M., Willems, G., Motte, P., Arfvidsson, S., Saumitou-Laprade, P., and Verbruggen, N., submitted to *Plant Journal* The major QTL for cadmium tolerance in *Arabidopsis halleri* co-localizes with *HMA4*, a gene encoding a Heavy Metal ATPase.

de Knecht, J. A., van Baren, N., Ten Bookum, W. M., Wong Fong Sang, H. W., Koevoets, P. L. M., Schat, H., *et al.*, 1995 Synthesis and degradation of phytochelatins in cadmium-sensitive and cadmium-tolerant *Silene vulgaris*. *Plant Science* **106:** 9-18.

Desbrosses-Fonrouge, A.-G., Voigt, K., Schröder, A., Arrivault, S., Thomine, S., and Krämer, U., 2005 *Arabidopsis thaliana* MTP1 is a Zn transporter in the vacuolar membrane which mediates Zn detoxification and drives leaf Zn accumulation. *FEBS Letters* **579:** 4165-4174.

Dräger, D. B., Desbrosses-Fonrouge, A.-G., Krach, C., Chardonnens, A. N., Meyer, R. C., Saumitou-Laprade, P., *et al.*, 2004 Two genes encoding *Arabidopsis halleri* MTP1 metal transport proteins co-segregate with zinc tolerance and account for high *MTP1* transcript levels. *Plant Journal* **39:** 425-439.

Guerinot, M. L., 2000 The ZIP family of metal transporters. *Biochimica et Biophysica Acta* **1465:** 190-198.

Hartley-Whitaker, J., Ainsworth, G., Vooijs, R., Ten Bookum, W., Schat, H., and Meharg, A. A., 2001 Phytochelatins are involved in differential arsenate tolerance in *Holcus lanatus*. *Plant Physiology* **126:** 299-306.

Hussain, D., Haydon, M. J., Wang, Y., Wong, E., Sherson, S. M., Young, J., *et al.*, 2004 P-Type ATPase Heavy Metal Transporters with roles in essential zinc homeostasis in Arabidopsis. *Plant Cell* **16:** 1327-1339.

Macnair, M. R., 1993 Tansley review No. 49: The genetics of metal tolerance in vascular plants. *New Phytologist* **124:** 541-559.

Macnair, M. R., Tilstone, G. H., and Smith, S. E., 2000 The genetics of metal tolerance and accumulation in higher plants, pp. 235-250 in *Phytoremediation of contaminated soil and water*, edited by C. Press.

Mills, R., Krijger, G., Baccarini, P., Hall, J. L., and Williams, L., 2003 Functional expression of AtHMA4, a P_{1B}-type ATPase of the Zn/Co/Cd/Pb subclass. *Plant Journal* **35:** 164-176.

Mills, R. F., Francini, A., Ferreira da Rocha, P. S., Baccarini, P. J., Aylett, M., Krijger, G. C., *et al.*, 2005 The plant P1B-type ATPase AtHMA4 transports Zn and Cd and plays a role in detoxification of transition metals supplied at elevated levels. *FEBS Letters* **579:** 783-791.

Papoyan, A., and Kochian, L. V., 2004 Identification of *Thlaspi caerulescens* genes that may be involved in heavy metal hyperaccumulation and tolerance. Characterization of a novel heavy metal transporting ATPase. *Plant Physiology* **136:** 3814-3823.

Sanito di Toppi, L., and Gabbrielli, R., 1999 Response to cadmium in higher plants. *Environmental and Experimental Botany* **41:** 105-130.

SAS, 2001 SAS user's guide: statistics, version 8.2. SAS Institute Inc., Cary, NC.

Schat, H., and Ten Bookum, W. M., 1992 Genetic control of copper tolerance in *Silene vulgaris*. *Heredity* **68:** 219-229.

Schat, H., and Vooijs, R., 1997 Multiple tolerance and co-tolerance to heavy metals in *Silene vulgaris*: a co-segregation analysis. *New Phytologist* **136:** 489-496.

Schat, H., Llugany, M., Vooijs, R., Hartley-Whitaker, J., and Bleeker, P. M., 2002 The role of phytochelatins in constitutive and adaptive heavy metal tolerances in hyperaccumulator and non-hyperaccumulator metallophytes. *Journal of Experimental Botany* **53:** 2381-2392.

Thomine, S., Wang, R., Ward, J. M., Crawford, N. M., and Schroeder, J. I., 2000 Cadmium and iron transport by members of a plant metal transporter family in Arabidopsis with homology to Nramp genes. *Proceedings of the National Academy of Sciences of the U S A* **97:** 4991-4996.

Van Ooijen, J. W., Boer, M. P., Jansen, R. C., and Maliepaard, C., 2002 MapQTL 4.0, Software for the calculation of QTL positions on genetic maps Plant Research International, Wageningen, the Netherlands.

Verret, F., Gravot, A., Auroy, P., Leonhardt, N., David, P., Nussaume, L., *et al.*, 2004 Overexpression of AtHMA4 enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. *FEBS Letters* **576:** 306-312.

Verret, F., Gravot, A., Auroy, P., Preveral, S., Forestier, C., Vavasseur, A., *et al.*, 2005 Heavy metal transport by AtHMA4 involves the N-terminal degenerated metal binding domain and the C-terminal His11 stretch. *FEBS Letters* **579:** 1515-1522.

Voorrips, R. E., 2002 Mapchart: Software for the graphical presentation of linkage maps and QTLs. *Heredity* **93:** 77-78.

Willems, G., Godé, C., Dräger, D., Courbot, M., Verbruggen, N., and Saumitou-Laprade, P., submitted to *Genetics* Quantitative Trait Loci mapping of zinc tolerance in the metallophyte *Arabidopsis halleri* ssp. *halleri.*

6 IDENTIFICATION OF POSITIONAL CANDIDATE GENES CONFERRING ZINC TOLERANCE IN *ARABIDOPSIS HALLERI* THROUGH THE COMBINATION OF TRANSCRIPTOME AND QUANTITATIVE TRAIT LOCI MAPPING ANALYSES

Willems, G., Mari, S.,Lebrun, M. and Saumitou-Laprade, P.

Article in preparation

6.1 Introduction

The genetic mechanisms that organisms deploy in order to cope with specific and sometimes, toxic growth conditions are of particular interest in evolutionary biology, since the understanding of these mechanisms offers fundamental insights into the nature of environmental adaptations. The fitness of an individual defined as its ability to survive and reproduce in specific environmental conditions is the result, at the biochemical level, of the specific regulation and/or expression of proteins (Feder and Walser 2005). Transcriptomics or transcriptional profiling consists in a genome-wide analysis of gene expression and is believed very promising in identifying those genes whose expression is regulated in relation to changes in the performance of the organism (Gracey and Cossins 2003). Transcriptomics have been used successfully in the study of adaptive responses of unicellular or more complex organisms (DeRisi *et al.* 1997; Gracey *et al.* 2004). However, there exists no simple relationship between the phenotype of an organism and its gene expression level. A differential genetic expression could be the cause as well as the consequence of the adaptive response showed by an organism (Liu *et al.* 2001). Moreover, a large numbers of genes are generally revealed through transcription profiling approaches, which makes it impossible to pursue a detailed functional analysis.

Arabidopsis halleri (n = 8) is a self-incompatible, outcrossing species of the Brassiceae family. This species is together with *A. lyrata* (n = 8) the closest wild relative of the model species *A. thaliana* (n = 5) (Mitchell-Olds 2001). Unlike its close relatives, *A. halleri* developed high zinc (Zn) and cadmium (Cd) tolerance, which allowed the species to efficiently cope with important heavy metal concentrations in the soil, occurring either naturally or as a consequence of human activities (Macnair *et al.* 1999). These metal tolerances are constitutive in *A. halleri* (Macnair *et al.* 1999; Bert *et al.* 2003), though minor quantitative differences in Zn tolerance have been observed between metallicolous and non-metallicolous *A. halleri* populations (Bert *et al.* 2000; Pauwels *et al.* 2005). It has been suggested that in addition to the background constitutive Zn tolerance level natural selection increased Zn tolerance in the *A. halleri* individuals from metalliferous sites (Bert *et al.* 2000).

Diverse strategies ranging from functional genomic approaches to high-throughput microarray analyses have been adopted to identify the genetic and physiological mechanisms underlying heavy metal tolerance. Circumstantial evidence for the implication in metal tolerance has been provided for only a small number of genes, such as the cation diffusion facilitator protein *MTP1* in Zn tolerance in *A. halleri* (Dräger *et al.* 2004) or phytochelatin synthase in arsenate tolerance in *Holcus lanatus* (Hartley-Whitaker *et al.* 2001). In a previous study we reported on the genetic architecture of Zn (Willems *et al.* submitted) and Cd tolerance (Courbot *et al.* submitted) in the *A. halleri* species by performing a Quantitative Trait Loci (QTL) analysis using an interspecific *A. halleri* x *A. l. petraea* first generation backcross (BC1) progeny. A genetic linkage map composed of eight linkage groups corresponding to the haploid chromosome number of *A. halleri* and *A. l. petraea* was constructed through the use of predominantly (75%) sequence-based markers anchored in the *A. thaliana* genome sequence (Willems *et al.* submitted). Three QTLs conferring Zn tolerance (Zntol-1, Zntol-2 and Zntol-3) (Willems *et al.* submitted) as well as three QTLs conferring Cd tolerance (Courbot *et al.* submitted) were identified on the linkage map. Based on these results a genetic model for Zn tolerance in *A. halleri* consisting of three genes with equal effects was suggested. For Cd tolerance the QTL Cdtol-1 was supposed to be the major one; the remaining QTLs exhibited less strong

effects. Three potential candidate genes for Zn tolerance (*HMA4*, *MTP1-A* and *MTP1-B*) and one potential candidate gene (*HMA4*) for Cd tolerance were proposed. However, their implication in the trait of interests could not yet be confirmed with certainty (Courbot *et al.* submitted; Willems *et al.* submitted).

In this study we have performed a suppression subtractive hybridisation (SSH) experiment on Zn-tolerant and sensitive individuals to identify genes differentially expressed in the roots and the leaves of the former compared to the latter. The SSH method is believed to generate a representation of differentially expressed genes irrespective of their relative abundance. This is particularly useful for the isolation of rare transcripts that might be undetected in other transcription profiling methods such as microarray analyses (Gracey and Cossins 2003). Moreover, the SSH method allows the isolation of differentially expressed cDNAs without an *a priori* knowledge of their sequence and is therefore appropriate for the study of gene expression in organisms for which genome information is scarce (Diatchenko *et al.* 1996; Sagerström *et al.* 1997; Gracey and Cossins 2003; Munir *et al.* 2004). The individuals used in this study originated from a first generation backcross progeny (BC1) developed on a cross between an individual of the Zn-tolerant and hyperaccumulating species *A. halleri* ssp. *halleri* and an individual of the non-tolerant, non-accumulating species *A. lyrata* ssp. *petraea*. Furthermore, we have compared the results obtained by the SSH experiment with those reported by the previously published microarray analyses conducted on *A. halleri* and *A. thaliana* growing in similar Zn conditions using *A. thaliana* GeneChips (Weber *et al.* 2004) (Becher *et al.* 2004). Additionally, we have integrated the results obtained in both transcriptome analyses and those obtained in the QTL analysis of Zn tolerance in *A. halleri* to identify positional candidate genes for Zn tolerance.

6.2 Materials and methods

Plant material and cultivation

An *A. halleri* x *A. l. petraea* BC1 progeny segregating for Zn and Cd tolerance was developed as described in Willems *et al.* (submitted). The evaluation of Zn tolerance of the 199 BC1 individuals was performed through a sequential exposure test according to Schat and Ten Bookum (1992) (Willems *et al.* submitted). Among those 199 individuals 23 BC1 individuals showing the highest Zn tolerance levels and 23 BC1 individuals showing the lowest Zn tolerance levels were selected for the SSH experiment. The 46 BC1 individuals were grown in a hydroponic solution to which 10 and 1000 µM of Zn was added as $ZnSO_4$ for the Zn-sensitive and Zn-tolerant BC1 individuals respectively. The nutrient solution was composed of 0.5 mM $Ca(NO_3)$, 0.2 mM $MgSO_4$, 0.5 mM KNO_3, 0.1 mM K_2HPO_4, 0.2 µM $CuSO_4$, 2 µM $MnCl_2$, 10 µM H_3BO_3, 0.1 µM MoO_3, 10 µM FeEDDHA and the pH of the solution was set at 6.5. After five days the roots and the leaves of each individual were harvested and used for RNA extraction.

Suppression subtractive hybridization

The SSH experiment was performed using the PCR-Select^{TM} cDNA Subtraction Kit (Clontech Laboratories, Inc.) following the manufacturer's instruction. The mRNAs of the Zn-tolerant and Zn-sensitive BC1 genotypes were used as tester and driver respectively to isolate only those genes that are differentially expressed in the tolerant genotypes compared to the sensitive ones. In a first step tester and driver cDNAs were digested with *Rsa*I. Following the digestion the tester cDNAs were subdivided in two populations and ligation of the tester cDNAs was performed with distinct adapter sequences for both samples. Two hybridisations were then performed. In the first hybridisation each tester cDNA was allowed to hybridise with an excess amount of driver cDNAs to subtract all cDNAs that were present in both tester and driver. Upon denaturation and annealing only the cDNAs specific to the tester population remained as single strands. Additionally, the concentration of high and low abundant sequences in the tester sample was supposed to be equalised because reannealing has proved to be faster for the more abundant cDNAs compared to those that are less abundant. In the following hybridisation step both tester populations were mixed together without denaturation. Consequently, only the cDNAs

present under a single-stranded conformation in each tester sample could associate. Two consecutive PCR amplifications were performed on the entire population of cDNAs. Primers with a sequence complementary to the adapter sequences were used to enrich the cDNA population in sequences specific to the tester. Only those double-stranded cDNAs originating from the hybridisation of two single-stranded cDNAs from both tester samples were consequently amplified exponentially. The cDNAs were then inserted into T/A-cloning vectors and all cDNA clones were sequenced. Sequences were submitted for BLAST N analysis using the *A. thaliana* cDNA database provided by a local server of the University of Montpellier 2 (France).

Comparative analysis of the *A. halleri* x *A. l. petraea*, the *A. l. petraea*, the *A. l. lyrata* and the *A. thaliana* maps

The chromosomal position (Megabases (Mb)) in *A. thaliana* of the markers used in the three different mapping experiments was obtained using the *A. thaliana* database (www.tair.org). Chromosomal rearrangements between *A. thaliana* and its wild relatives *A. halleri*, *A. l. petraea* and *A. l. lyrata* within or near the Zn tolerance QTLs were identified to ensure a correct transfer of the QTL confidence intervals from the *A. halleri* x *A. l. petraea* map (henceforth *Ah* x *Alp* map) to the *A. thaliana* map.

Identification of positional candidate genes

We obtained the position of the *A. thaliana* homologs identified in the SSH experiment and the microarray analyses (Becher *et al.* 2004; Weber *et al.* 2004) on the *A. thaliana* database (www.tair.org). A physical *A. thaliana* map on which the genes identified in the SSH experiment and the microarrays were positioned, was constructed using Mapchart (Voorrips 2002). According to the results of the comparative analysis conducted on the different maps the QTL regions conferring Zn tolerance were transferred to the *A. thaliana* physical map. To identify positional candidate genes we verified the co-localisation of the differentially expressed genes with the QTL regions for Zn tolerance.

6.3 Results

Comparative analysis of the *A. halleri* x *A. l. petraea*, the *A. l. petraea*, the *A. l. lyrata* and the *A. thaliana* maps

The comparative analysis was restricted to those linkage groups of the *Ah* x *Alp* map on which the QTLs for Zn tolerance were detected, i.e. LG3, LG4 and LG6 (Willems *et al.* submitted) (Figure 6.1; Table 6.1).

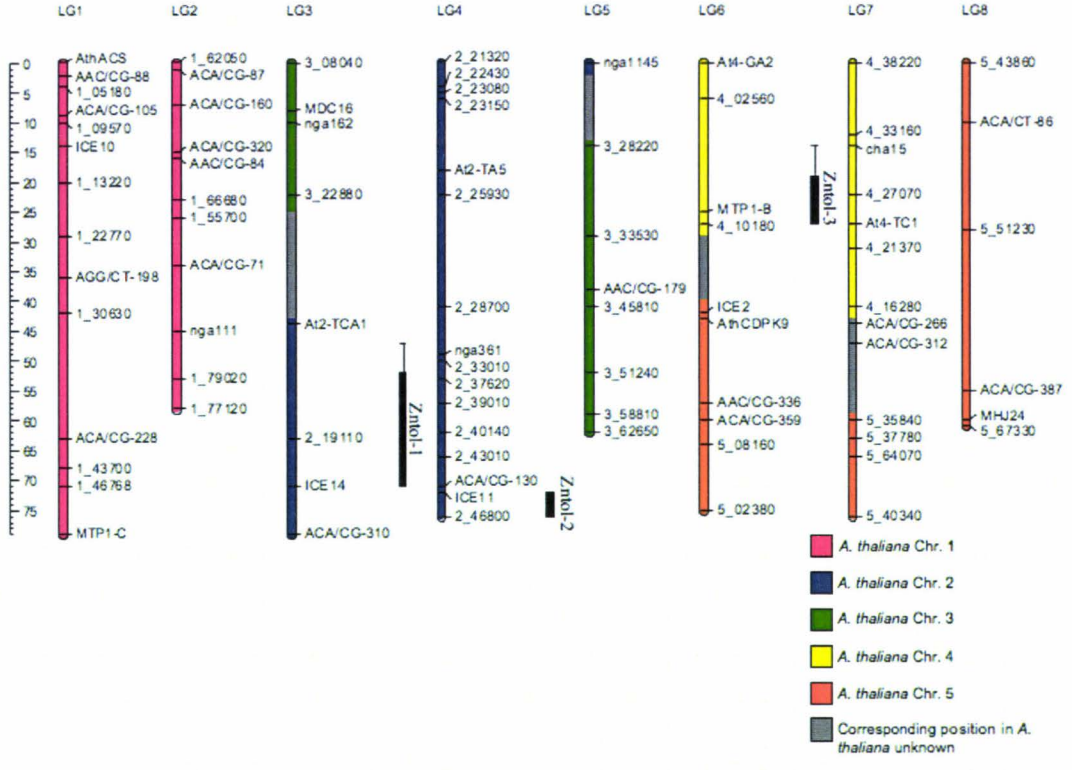


Figure 6.1 Genetic linkage map constructed on the *A. halleri* x *A. l. petraea* BC1 progeny and position of the QTLs for Zn tolerance, as described in Willems *et al.* (submitted).

Sequence-based markers anchored in *A. thaliana* have been named according to the *A. thaliana* homolog in which the marker was defined (ex. on LG1 marker 1_05180 defined within the gene *At1g05180*). One- (filled black bars) and two-LOD support intervals (whiskers extending beyond the bars) associated with the QTLs for Zn tolerance (Zntol-1, Zntol-2 and Zntol-3) are indicated at the right of the linkage groups.
The ruler at the left of the figure indicates the position (in centiMorgan) of the markers on the linkage map. The colours indicate the corresponding *A. thaliana* chromosome.

In the comparative analysis between the *Ah* x *Alp* and the *A. thaliana* maps we included the mapping experiments recently published on *A. l. petraea* (Kuittinen *et al.* 2004) and *A. l. lyrata* (Yogeeswaran *et al.* 2005) (Table 6.1). This should provide us a more extensive view of all chromosomal rearrangements that occurred since the divergence between *A. thaliana* and its close relatives *A. halleri* and *A. lyrata*, which is essential if we want to avoid an erroneous transfer of the QTL regions to the *A. thaliana* physical map.

Table 6.1 Linkage groups on which the QTLs for Zn tolerance were detected in the *A. halleri* x *A. l. petraea* map and their homolog in the *A. l. petraea*, *A. l. lyrata* and *A. thaliana* maps

QTLs for Zn tolerance	*Ah* x *Alp* map	*A. l. petraea* map	*A. l. lyrata* map	*A. thaliana* map
Zntol-1	LG3	AL3	AlyLG4	At_Chr.II
Zntol-2	LG4	AL4	AlyLG3	At_Chr.II
Zntol-3	LG6	AL6	AlyLG7	At_Chr.IV

With regard to the QTL Zntol-1 no rearrangements were observed between the *Ah* x *Alp*, the *A. lyrata* maps and the corresponding *A. thaliana* genomic region (Figure 6.2). As previously reported in the three mapping experiments conducted on the Arabidopsis relatives (Kuittinen *et al.* 2004; Yogeeswaran *et al.* 2005; Willems *et al.* submitted), a reciprocal translocation occurred between the linkage groups LG3 and LG4 of the *Ah* x *Alp* map. The QTL region detected on the linkage group LG3 however, was not concerned by this event.

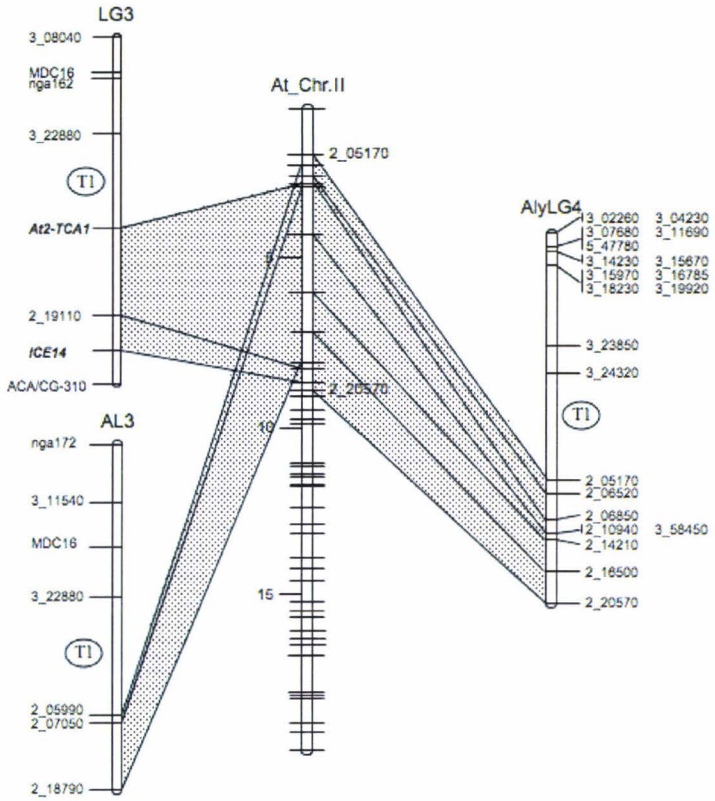


Figure 6.2 Identification of the chromosomal rearrangements in the QTL region Zntol-1 through a comparative analysis of the *Ah* x *Alp* (LG3), the *A. l. petraea* (AL3), the *A. l. lyrata* (AlyLG4) and the *A. thaliana* (At_Chr.II) maps.

Markers defining the QTL region Zntol-1 on the *Ah* x *Alp* map (Willems *et al.* submitted) are indicated in bold and italic. T1 indicates the approximate position of the breakpoint that involved a translocation event between the linkage groups LG3 and LG4 on the *Ah* x *Alp* map (also observed for the *A. lyrata* subspecies).

On the linkage group AlyLG3 of the *A. l. lyrata* map a minor inversion was reported in the upper part of the linkage group between the marker loci *2_28870* and *2_27530* (Yogeeswaran *et al.* 2005). The QTL region Zntol-2 detected on the corresponding linkage group LG4 in the *Ah* x *Alp* map (Willems *et al.* submitted) was not concerned by this event because the QTL was localised on the lower arm of LG4 (Figure 6.3).

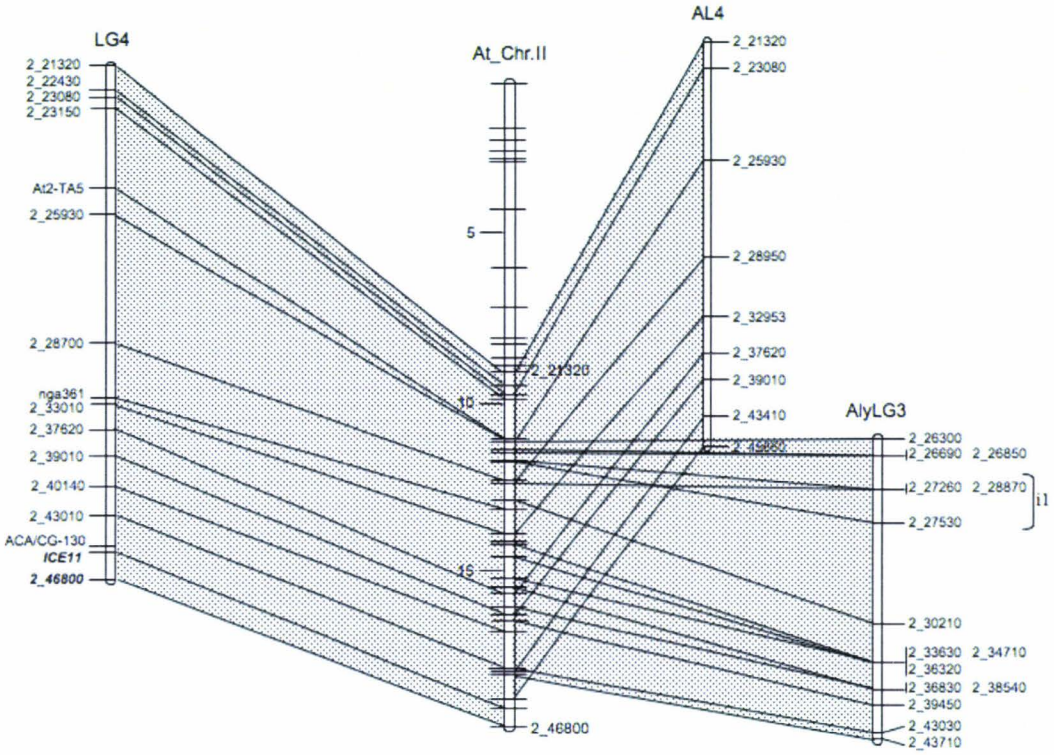


Figure 6.3 Identification of the chromosomal rearrangements in the QTL region Zntol-2 through a comparative analysis of the *Ah* x *Alp* (LG4), the *A. l. petraea* (AL4), the *A. l. lyrata* (AlyLG3) and the *A. thaliana* maps (At_Chr.II).

Markers defining the QTL region Zntol-2 on the *Ah* x *Alp* map (Willems *et al.* submitted) are indicated in bold and italic. i1 indicates the inversion observed on AlyLG3 (*A. l. lyrata*).

One large inversion was identified in *A. l. petraea* on the upper arm of the linkage group AL6 (Kuittinen *et al.* 2004) and in *A. l. lyrata* on the corresponding linkage group AlyLG7 (Yogeeswaran *et al.* 2005). The inverted region co-localised with the QTL region Zntol-3 identified on the linkage group LG6 in the *Ah* x *Alp* map (Willems *et al.* submitted) (Figure 6.4). This inversion might represent the ancestral state of marker order (Yogeeswaran *et al.* 2005) since the same event was observed in *Capsella rubella* (Boivin *et al.* 2004), another wild relative of *A. thaliana*. Thus, the inversion necessary to give rise to the order in *A. thaliana* should have occurred in the *A. thaliana* lineage after its divergence from the *A. lyrata* and *A. halleri* species. On the *Ah* x *Alp* map this inversion was not detected probably because of the low density of the markers in this region. However, a duplication event was identified in this region on the *Ah* x *Alp* map (Dräger *et al.* 2004; Willems *et al.* submitted). The marker locus *MTP1-B* was one of the two

paralogs of the *MTP1* gene. This gene was mapped on LG4 on the *Ah* x *Alp* map as expected from the position of the *A thaliana* homolog *MTP1* on At_Chr.II. The second paralog *MTP1-C* mapped on LG1 on the *Ah* x *Alp* map (Figure 6.1). This duplication event was limited to *A. halleri* and was not observed in the closely related species *A. l. petraea* (Dräger *et al.* 2004). A second small inversion was reported on the linkage group AL6 on the *A. l. petraea* map (Kuittinen *et al.* 2004), though not confirmed with certainty by the authors. A second translocation event involved the linkage groups LG6 and LG7 of the *Ah* x *Alp* map. The QTL Zntol-3 on LG6 was located near the breaking point.

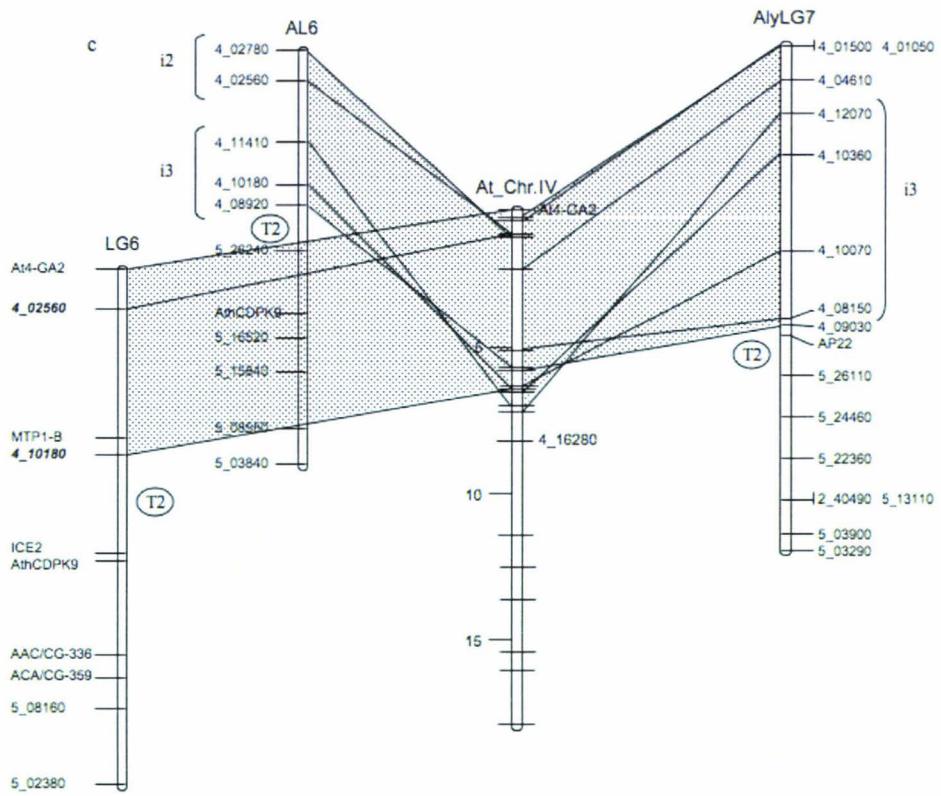


Figure 6.4 Identification of the chromosomal rearrangements in the QTL region Zntol-3 through a comparative analysis of the *Ah* x *Alp* (LG6), the *A. l. petraea* (AL6), the *A. l. lyrata* (AlyLG7) and the *A. thaliana* maps (At_Chr.IV).

Markers defining the QTL region Zntol-3 on the *Ah* x *Alp* map (Willems *et al.* submitted) are indicated in bold and italic. i2 and i3 indicate the inversions observed on AL6 (*A. l. petraea*) and, AL6 (*A. l. petraea*) and AlyLG3 (*A. l. lyrata*) respectively. T2 indicates the approximate position of the breakpoint that involved a translocation event between the linkage groups LG6 and LG7 on the *Ah* x *Alp* map (also observed for the *A. lyrata* subspecies).

Transfer of the QTL regions for Zn tolerance from the *A. halleri* x *A. l. petraea* to the *A. thaliana* map

On the *Ah* x *Alp* map the markers *At2-TCA1* and *ICE14* and the markers *ICE11* and *2_46800* delimited the QTL regions Zntol-1 and Zntol-2 respectively. Since no rearrangements were observed in these regions (Figure 6.3; Figure 6.4) they could be readily transferred to *A. thaliana*. In *A. thaliana* these two QTL regions mapped to the second chromosome. The QTL region Zntol-1 was situated in the upper part of the chromosome, whereas Zntol-2 was positioned on the extremity of the lower arm of At_Chr.II. The marker *4_02560* was adopted to define the upper limit of the QTL region Zntol-3 in *A. thaliana* because in the QTL mapping experiment we did not have any other marker anchored in *A. thaliana* nearer the upper limit of the QTL region Zntol-3. The lower limit of the QTL region corresponded to the marker *4_10180* on the *Ah* x *Alp* map. This marker however, could not be used to define the lower limit of the QTL region in *A. thaliana* since an inversion in marker order in this region most probably occurred, as was reported for the *A. lyrata* subspecies (Kuittinen *et al.* 2004; Yogeeswaran *et al.* 2005). The lower limit of the QTL region was therefore inferred from the breakpoint on LG6 that was involved in the reciprocal translocation between the linkage groups LG6 and LG7 (Figure 6.4). Although the exact position of the breakpoint could not be predicted due to the low resolution of the different genetic linkage maps, we could infer the approximate position by integrating the information obtained in the different mapping experiments. The mapping results indicated that the breakage should have occurred between the markers *4_12070*, positioned on AlyLG7 of the *A. l. lyrata* map, and *4_16280* located on LG7 and AL7 in the *Ah* x *Alp* map and the *A. l. petraea* map respectively. The marker *4_16280* was consequently used to define the lower limit of the QTL region Zntol-3 in *A. thaliana*.

Isolation of differentially expressed genes in Zn-tolerant *A. halleri* x *A. l. petraea* individuals through a Suppression Subtractive Hybridisation experiment

We isolated 431 cDNAs supposed to be differentially expressed in the Zn- tolerant BC1 individuals compared to their Zn-sensitive relatives. Among these sequences 40% (173) were identified in the roots, the remaining 60% (258) were identified in the leaves. For five cDNAs, identified in the leaves, no homologous *A. thaliana* genes could be found.

Additionally, 11 sequences isolated in the leaves too showed homology at a same degree with more than one *A. thaliana* homolog. These 16 cDNAs were consequently excluded from the following analyses. By performing a BLAST analysis on the remaining 415 cDNA sequences 253 *A thaliana* homologs were identified. For the roots and the leaves respectively 73 and 181 *A. thaliana* homologs were detected; one gene was identified in both samples. A significantly lower number of *A. thaliana* homologs was detected when compared to the number of sequences isolated. This relies on the fact that 63 *A. thaliana* homologs (31 for the roots, 32 for the leaves) were identified more than once in the BLAST analysis. The majority of these genes (36 or 57%) were isolated twice in either the root or the leaf samples (Figure 6.5).

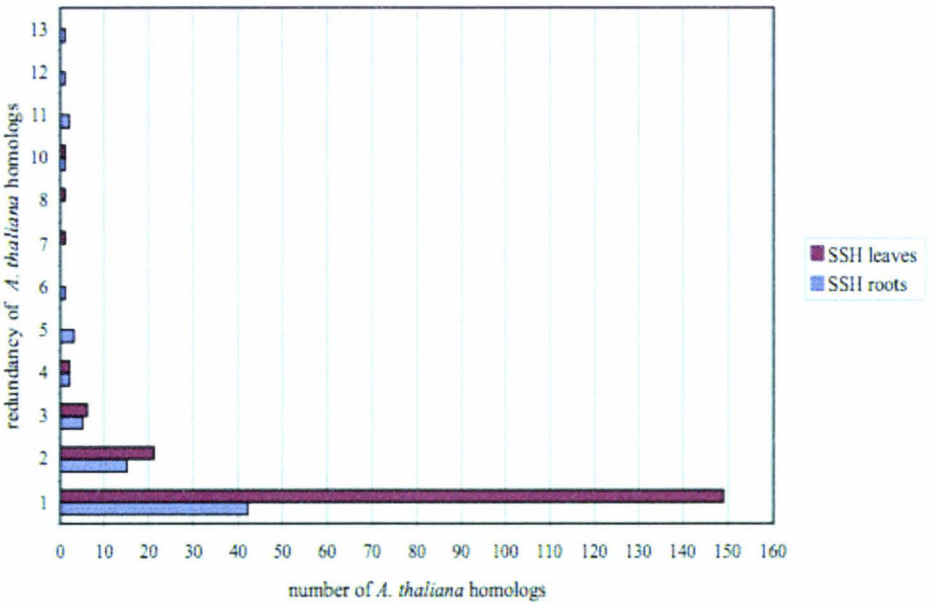


Figure 6.5 Graphical representation of the number of *A. thaliana* genes that were identified for 1 to 13 times in the SSH experiment.

On the X-axis the number of different *A. thaliana* homologs is indicated; the Y-axis indicates the frequency with which the *A. thaliana* homologs were identified. The genes identified in the leaf sample of the SSH experiment are indicated in red. The genes identified in the root sample of the SSH experiment are indicated in blue.

Among the *A. thaliana* homologs identified through the BLAST analysis 27% were of unknown function (21 and 49 genes in the roots and leaves respectively), 23% were predicted to be involved in the general metabolism (lysis, biosynthesis and modification of proteins, lipids and carbohydrates) (16 and 41 genes isolated in the roots and leaves

respectively), 11% in photosynthesis (1 and 27 genes isolated in the roots and leaves respectively), 11% in stress protection or pathogen defence (8 and 21 genes isolated in the roots and leaves respectively), 7% in transport (5 and 12 genes isolated in the roots and leaves respectively), 6% in transcription and translation (8 and 6 genes isolated in the roots and leaves respectively) and 8% in other functions (RNA/DNA binding, cell wall biosynthesis, microtubule based processes) (6 and 15 genes isolated in the roots and leaves respectively) (Figure 6.6). Among all *A. thaliana* homologs 18 or 7% were expected to be involved in metal homeostasis or to be metal-dependent (Figure 6.6; Table 6.2). In *A. thaliana* these 18 genes encoded two embryo-abundant proteins, several zinc-, copper- and iron-binding proteins, four metal transporters, one metal chelator and a Cu/Zn binding superoxide dismutase (Table 6.2).

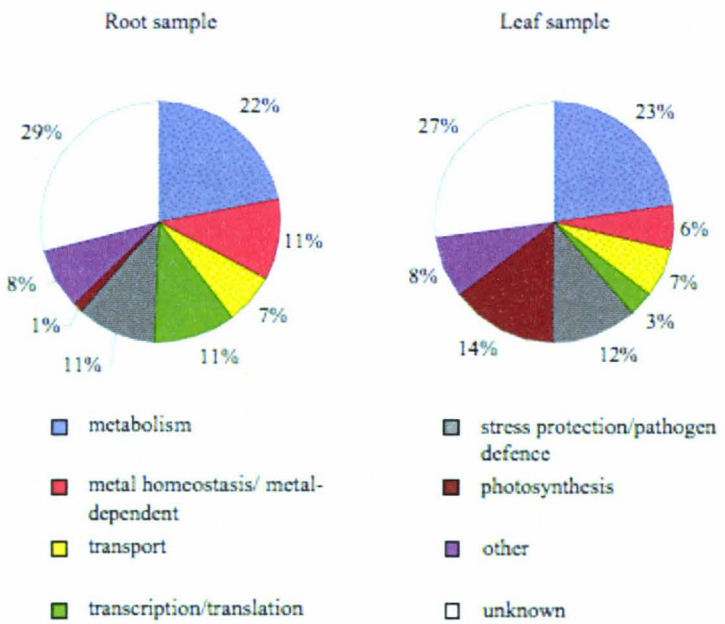


Figure 6.6 Functional classification of differentially expressed genes in the root and the leaf sample of the SSH experiment.

The percentage of genes identified in each of the functional classes is indicated.

TABLE 6.2 Differentially expressed genes involved in metal homeostasis and/or interacting with metals

AGI code[a]	Annotation[b]	Experiment[c]
Fe homeostasis		
At2g41380	embryo-abundant related protein	leaves
At5g10830	embryo-abundant related protein	roots
Metal transport		
At1g15960	NRAMP metal ion transporter 6, putative (NRAMP6)	roots
At1g59870	ABC transporter family protein	leaves
At3g56940	putative ZIP protein	leaves
At5g60790	ABC transporter family protein	leaves
At3g09390	metallothionein-related protein (MT2a)	leaves
Zn-dependent proteins		
At1g23740	oxidoreductase, dehydrogenase family	leaves
At2g26140	FtsH protease, putative	roots
At2g40140	CCCH-type zinc finger protein-related	roots
At3g05200	RING-H2 zinc finger protein ATL6-related	roots
At3g19910	zinc finger (C3HC4-type RING finger) protein family	roots
At5g04340	C2H2 zinc finger transcription factor-related	roots
At5g57660	CONSTANS B-box zinc finger family protein	leaves and roots
At5g61510	putative NADP-dependent oxidoreductase	leaves
Cu-dependent protein		
At3g43670	copper amine oxidase-related protein	leaves
Cu/Zn-dependent protein		
At2g28190	copper/zinc superoxide dismutase (CSD2)	leaves
Fe-dependent protein		
At5g05600	oxidoreductase, 2OG-Fe(II) oxygenase	leaves

[a]AGI code obtained in the BLAST analysis
[b]Annotation of the gene
[c]SSH sample in which the gene was isolated

Comparative analysis of the genes isolated through the Suppression Subtractive Hybridisation experiment and the microarray analyses

A physical *A. thaliana* map was constructed on which the differentially expressed genes of the SSH experiment (253 genes) and the microarray analyses (72 genes) (Becher *et al.* 2004; Weber *et al.* 2004) were positioned (Figure 6.7). Four genes *At2g43590*, *At3g12500*, *At3g16460* and *At5g24780* were isolated in both transcriptome analyses. With the exception of *At3g12500* these genes were all isolated in the leaf samples of both

transcriptome experiments. The *A. thaliana* homolog *At3g12500* was identified in the root sample of the SSH experiment and in the leaf sample of the microarray analysis. With the exception of *At3g16460* with an unknown biological function, these genes are all involved in stress protection and pathogen defence.

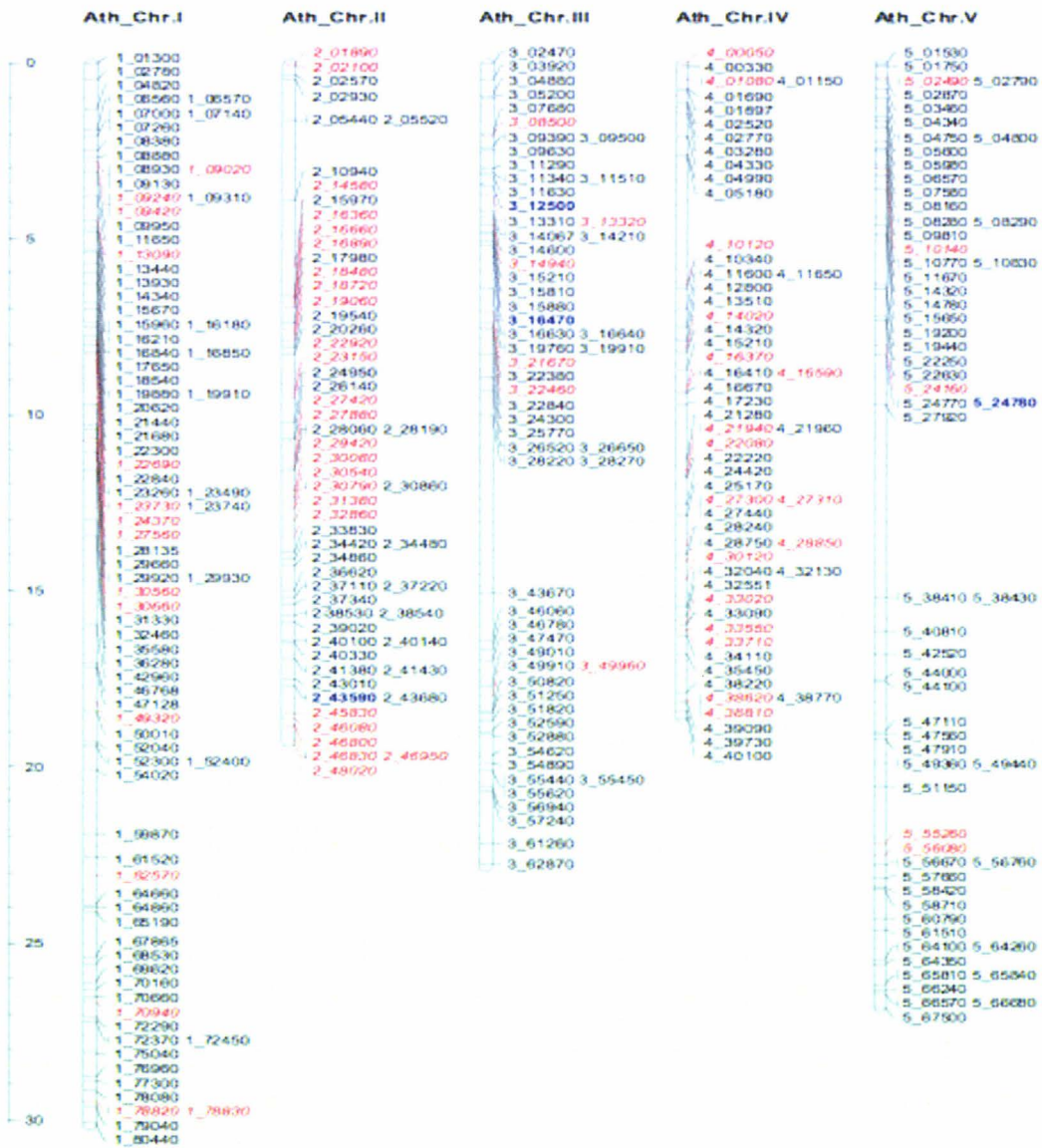


FIGURE 6.7 *A. thaliana* physical map on which the differentially expressed genes isolated in the SSH experiment and the microarray analyses were positioned.

In black, genes isolated by the SSH experiment; in red and italic, genes identified by the microarrays; in blue, genes identified in both analyses. The ruler at the left of the figure indicates the position of the genes (in Mb).

Identification of positional candidate genes

Through the transfer of the QTL regions to the *A. thaliana* physical map we identified 26 genes among the total number of 325 genes isolated in both transcriptome analyses that co-localised with the QTL regions for Zn tolerance (Figure 6.8; Table 6.3). Eleven genes mapped to the QTL region Zntol-1: four of them were identified by the SSH experiment in the leaves, two genes were revealed by the microarray analysis in the root sample and five genes were identified by the microarray analysis in the leaves. One gene revealed by the microarray analysis in the leaves was positioned in the second QTL region. The remaining 14 genes co-localised with the QTL region Zntol-3: 12 genes were identified by the SSH experiment (10 genes in the leaves, 2 genes in the roots), two genes were revealed by the microarray analysis among which one was identified in the leaves and one in the roots. Among the 26 positional candidate genes, only a single one, *At2g46800*, was reported in the literature to be involved in metal homeostasis and more precisely in Zn homeostasis in *A. halleri*.

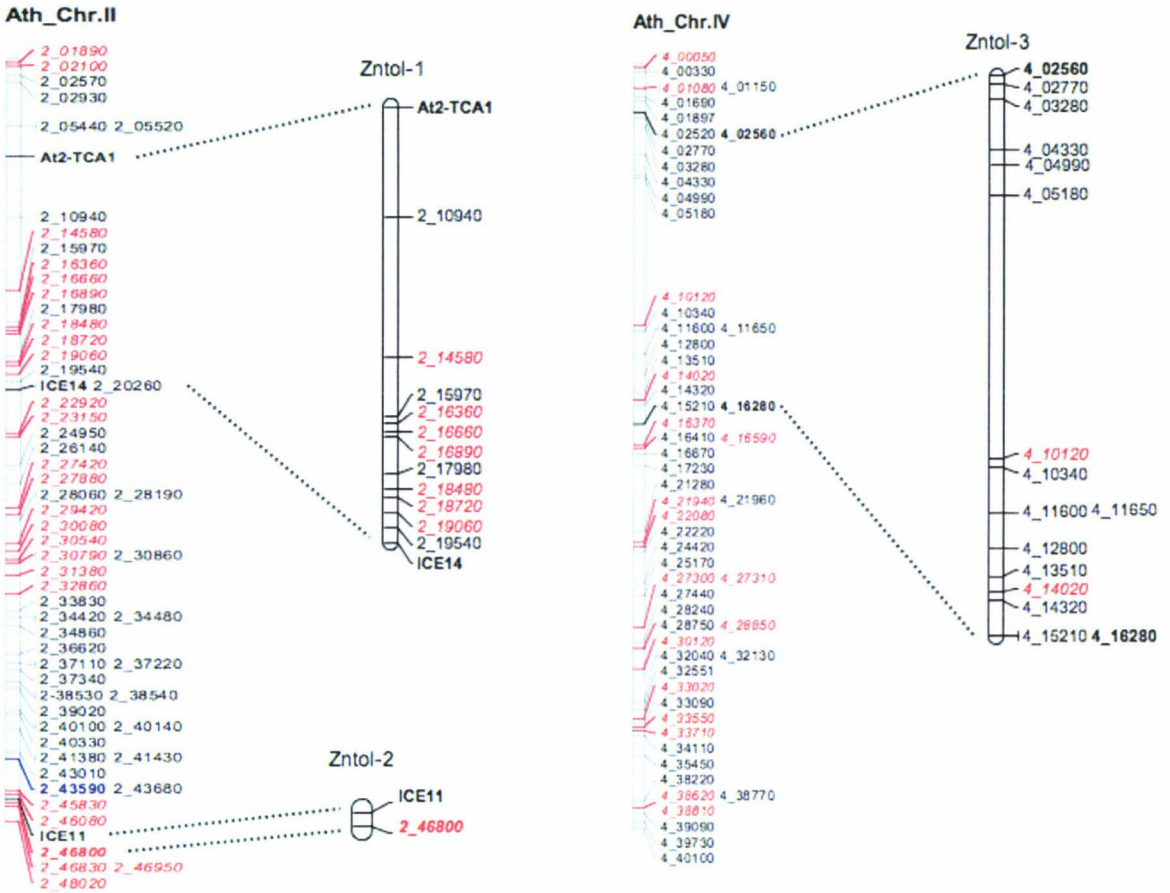


FIGURE 6.8 Identification of positional candidate genes for Zn tolerance through the analysis of the co-localisation of the differentially expressed genes with the QTL regions conferring Zn tolerance.

At the right of At_Chr.II and At_Chr.IV a detailed view is given of the QTL regions Zntol-1, -2 and -3 and the genes that are positioned within the QTL regions. In bold, the markers of the *Ah* x *Alp* map that define the QTL regions; in black, *A. thaliana* homologs identified by the SSH experiment; in red and italic, *A. thaliana* homologs identified by the microarrays.

TABLE 6.3 Positional candidate genes for Zn tolerance

AGI code[a]	Annotation[b]	Class[c]	Experiment[d]
Positional candidate genes for Zntol-1			
At2g10940	protease inhibitor/seed storage/lipid transfer protein	transport	SSH L
At2g14580	putative pathogenesis-related protein-like	stress/pathogen defence	microarray L
At2g15970	cold acclimation protein WCOR413 Triticum aestivum-related	stress/pathogen defence	SSH L
At2g16360	40S ribosomal protein S25	metabolism	microarray L R

TABLE 6.3 (continued)

AGI code[a]	Annotation[b]	Class[c]	Experiment[d]
At2g16660	nodulin-like protein, transporter	transport	microarray R
At2g16890	putative glucosyltransferase	metabolism	microarray L
At2g17980	SEC1 family transport protein-related	transport	SSH L
At2g18480	major facilitator superfamily, putative sugar transporter	transport	microarray L
At2g18720	putative translation initiation factore elF-2 gamma subunit	transcription/translation	microarray L
At2g19060	putative GDSL-motif lipase	metabolism	microarray R
At2g19540	transducin / WD-40 repeat protein family	unknown	SSH L
Positional candidate genes for Zntol-2			
At2g46800	cation diffusion facilitator family Zn transporter MTP1	metal homeostasis	microarray L
Positional candidate genes for Zntol-3			
At4g02770	photosystem I reaction center subunit II precursor	photosynthesis	SSH L
At4g03280	Rieske FeS protein (component of cytochrome B6-F complex)	photosynthesis	SSH L
At4g04330	expressed protein	unknown	SSH L
At4g04990	unknown protein, similar to Gossypium hirsutum cotton fiber expressed protein 1	unknown	SSH R
At4g05180	oxygen-evolving complex protein 16, chloroplast precursor	photosynthesis	SSH L
At4g10120	sucrose-phosphate synthase-like protein	metabolism	microarray L
At4g10340	light-harvesting chlorophyll a/b binding protein	photosynthesis	SSH L
At4g11600	glutathione peroxidase, putative	metabolism	SSH L
At4g11650	osmotin-like protein	stress/pathogen defence	SSH R
At4g12800	probable photosystem I chain XI precursor	photosynthesis	SSH L
At4g13510	ammonium transport protein	transport	SSH L
At4g14020	unknown protein	unknown	microarray R
At4g14320	60S ribosomal protein L36a/L44	metabolism	SSH L
At4g15210	glycosyl hydrolase family 14	stress/pathogen defence	SSH L

[a]AGI code obtained in the BLAST analysis
[b]Annotation of the gene
[c]Class of the gene according to its annotation
[d]Experiment in which the gene was isolated: microarray or SSH, L for the leaves, R for the roots

6.4 Discussion

Identification of differentially expressed genes in Zn-tolerant individuals originating from an *A. halleri* x *A. l. petraea* BC1 progeny through a Suppressive Subtractive Hybridisation experiment

Given the high number of cDNA clones and *A. thaliana* homologous genes identified through the SSH experiment we might consider that this method is a powerful means for the isolation of differentially expressed genes. However, verification of the differential expression of the genes identified in the SSH experiment through Northern Blot analysis has not been performed. Consequently, we might expect false positives to be present among the isolated sequences.

Through the BLAST analysis we observed that 25% of the *A. thaliana* homologs were identified more than once in the SSH experiment. Ideally, redundancy among the sequences should not occur since the hybridisation between single-stranded DNA is expected to occur more easily for abundant sequences compared to less abundant ones.

Only few *A. thaliana* homologs (18 or 7%) were expected to be involved in metal transport and metal chelation or were expected to interact with metal ions. Moreover, only a single one *At2g46800* was reported with a well-established function in Zn homeostasis in *A. thaliana* (Desbrosses-Fonrouge *et al.* 2005; Krämer 2005) and in Zn tolerance in *A. halleri* (Dräger *et al.* 2004). For this protein a role in vacuolar Zn sequestration was proposed (Dräger *et al.* 2004; Desbrosses-Fonrouge *et al.* 2005; Krämer 2005). In addition to MTP1 a role in Zn homeostasis might be expected for the putative ZIP protein, for NRAMP6 and MT2a. In *A. thaliana*, with the exception of the *IRT1* gene, all ZIP proteins are believed to be involved in the transport of various cations including Zn (Guerinot and Eide 1999; Guerinot 2000). The ZIP family counts 15 members, among which three have been functionally characterised in *A. thaliana*. The *ZIP1* and *ZIP3* genes may be involved in Zn uptake in the roots (Grotz *et al.* 1998), whereas for ZIP4 a role in Zn transport within the cell or between plant tissues was suggested (Guerinot and Eide 1999; Guerinot 2000). In the Zn-tolerant and hyperaccumulator species *Thlaspi caerulescens* the *AtZIP4* ortholog *ZNT1* might be responsible for Zn influx in the roots (Lasat *et al.* 2000; Pence *et al.* 2000).

Additionally, *TcZNT1* might be involved in Zn hyperaccumulation rather than in Zn tolerance, given that the same expression levels were observed for this gene in accessions exhibiting different tolerance levels (Assunçao *et al.* 2001). The *AtNRAMP6* gene has not yet been functionally characterised. A role in Fe and Mn transport might however be expected as has been suggested for *AtNRAMP1* because of the sequence homology between both genes (Hall and Williams 2003; Weber *et al.* 2004). The metal chelator protein MT2a was studied with regard to its role in copper (Cu) homeostasis in *A. thaliana* (Murphy and Taiz 1995; Zhou and Goldsbrough 1995) and in Cu-tolerant *Silene vulgaris* populations (van Hoof *et al.* 2001). However, in *A. thaliana* evidence was also provided for the interaction of *MT2* with Zn since an enhancement of the expression of *MT2* in *A. thaliana* was observed upon exposure to this metal (Murphy and Taiz 1995; Zhou and Goldsbrough 1995).

Comparative analysis of the genes isolated through the SSH experiment and the microarray analyses

The comparative analysis of the SSH experiment and the microarrays indicated (a) that a significantly higher number of differentially expressed genes was identified in the SSH experiment, (b) that a low number of genes were identified in both analyses. The differences observed between both analyses might rely on several factors. As mentioned above, the lack of verification of the differential expression of the cDNA clones identified in the SSH experiment through Northern Blot might have involved the presence of a high number of false positives. In addition, both analyses were not performed on the same plant material and under identical growth conditions. In the microarrays a Zn-tolerant *A. halleri* individual was compared with a Zn-sensitive *A. thaliana* individual to identify differentially, i.e. higher in this case, expressed genes in the former compared to the latter under low Zn concentrations (1 µM for the microarrays on the leaves, 0.8 µM for the microarrays on the roots) (Becher *et al.* 2004; Weber *et al.* 2004). Because two distinct species were compared a differential gene expression is not necessarily related to Zn tolerance, but might well be the consequence of the divergence time between both species. In the SSH experiment all individuals originated from an interspecific *A. halleri* x *A. l. petraea* BC1 progeny; they should consequently be identical throughout the genome except for the genes implicated in Zn tolerance. The Zn-tolerant individuals were on the

other hand exposed to significantly higher Zn concentrations (1 mM) than those used in the microarrays and than those to which the Zn-sensitive individuals were exposed (10 μM). However, an up- or downregulation of the gene expression in response to the metal concentration might occur and might be expected in the context of this study more particularly for genes involved in Zn homeostasis, as reported for instance for *AhMTP1* in the roots under high Zn conditions (Dräger *et al.* 2004). The microarray analyses were performed using *Arabidopsis* GeneChips that contained approximately one third (8300 genes) of the *A. thaliana* genome. Thus, the *A. thaliana* genome was not totally represented on the GeneChips and this might have contributed to the differences observed between both transcription profiling studies. Finally, in the microarrays only those genes were revealed that showed a eightfold higher expression in *A. halleri* compared to *A. thaliana* to avoid problems relative to a possible underestimation of the *A. halleri* gene expression because of sequence divergence between *A. halleri* and *A. thaliana*. However, the application of this threshold value might have involved that the genes for which the difference in expression between the two species was less important, remained undetected. Contrary to the microarrays the SSH method enables the identification of genes that are either lowly or highly expressed.

Identification of positional candidate genes

Transcription profiling methods are believed to be unable in distinguishing causal genes from those that are secondarily affected (Liu *et al.* 2001). Consequently, a large number of genes are usually revealed by these methods. A supplemental screen is consequently useful to identify those genes that are causal to the trait of interest, rather than differentially expressed as a consequence of the adaptation. This could for instance be performed by considering the function of the identified genes. However, this strategy has some disadvantages. Firstly, the function of many genes, even in the model species *A. thaliana* is not known. In the SSH experiment for instance 27% of the genes are of unknown function. Secondly, by applying such a strategy we necessarily restrict us to those genes for which a function in the trait of interest is already well established. This, however, is rather contradictory to the concept of transcription profiling methods through which one tends to identify genes that are not yet known to be involved in the trait under study. In this study we integrated the results obtained through transcription profiling

analyses and those obtained in the QTL analysis of Zn tolerance in *A. halleri* to identify only those genes for which the differential expression should be causative of Zn tolerance. By analysing the co-localisation of the differentially expressed genes with the QTL regions for Zn tolerance, we were able to provide a list of so-called "positional candidate genes" for Zn tolerance. In *Drosophila melanogaster* Wayne and McIntyre (2002) implemented a similar strategy for the identification of candidate genes for ovariole number. The co-localisation of differentially expressed genes revealed by microarray analysis with the QTL regions identified for ovariole number allowed to reduce significantly the number of candidate genes (Wayne and McIntyre 2002). Combined strategies integrating transcriptomics and QTL mapping methods have also been successfully applied in the identification of susceptibility loci underlying complex diseases (Altman *et al.* 1999; Liu *et al.* 2001; Eaves *et al.* 2002). We identified 26 genes that co-localised with one of the QTL regions for Zn tolerance. Only one of these genes, *At2g46800*, has a clearly established function in Zn homeostasis. This gene corresponds to *MTP1* and was intensively studied in *A. thaliana* (van der Zaal *et al.* 1999; Desbrosses-Fonrouge *et al.* 2005) and *A. halleri* (Dräger *et al.* 2004) with regard to its role in Zn tolerance. A role in vacuolar sequestration of Zn was suggested for MTP1 in *A. halleri* and *A. thaliana* (van der Zaal *et al.* 1999; Dräger *et al.* 2004; Desbrosses-Fonrouge *et al.* 2005).

An essential next step consists now in the genetic mapping of these genes on the *Ah* x *Alp* map in order to confirm their co-localisation with the QTLs. This was already accomplished for the gene *At2g46800*. The orthologous copy of this gene in *A. halleri* (*MTP1-A*) localised within the QTL region Zntol-2 as expected from the comparative analysis between the *Ah* x *Alp* linkage map and the *A. thaliana* genome (Willems *et al.* submitted). Since no chromosomal rearrangements were observed for the QTL region Zntol-1, we expect the genetic mapping of the positional candidate genes identified within this QTL region to confirm the position obtained through the physical mapping of these genes. In the case of the QTL region Zntol-3 however, the physical position deduced from the comparative analyses between the *Ah* x *Alp* and the *A. thaliana* maps might be different from the position that will be obtained through genetic mapping because an inversion was observed within this QTL region. Upon the confirmation of the co-localisation of the positional candidate genes with the QTLs conferring Zn tolerance as well as of their

segregation with Zn tolerance in the *Ah* x *Alp* BC1 progeny, the functional characterisation of these genes should be performed in order to establish their role in Zn tolerance.

In conclusion, transcription profiling methods are powerful in identifying differentially expressed genes by comparing individuals exhibiting contrasting phenotypes. One of the major limits inherent to these methods however, relates to the large number of genes that are usually revealed. The gene *At2g46800* identified through microarray analysis and reported to map at the QTL Zntol-2 supports our claim that linkage mapping of differentially expressed genes provides a useful screen of transcription profiling results for the identification of positional candidate genes. In turn, the genetic mapping of those genes results in an increase of the marker density in the QTL regions, that will allow us to reduce the confidence intervals associated with the QTL regions and consequently, to map the QTLs with more precision.

6.5 References

Altman, T. J., Glazier, A. M., Wallace, C. A., Cooper, L. D., Norsworthy, P. J., Wahid, F. N., *et al.*, 1999 Identification of Cd36 (Fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypersensitive rats. *Nature Genetics* **21:** 76-83.

Assunçao, A. G. L., da Costa Martins, P., de Folter, S., Vooijs, R., Schat, H., and Aarts, M. G. M., 2001 Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens*. *Plant Cell and Environment* **24:** 217-226.

Becher, M., Talke, I. N., Krall, L., and Krämer, U., 2004 Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. *Plant Journal* **37:** 251-268.

Bert, V., Macnair, M. R., de Laguerie, P., Saumitou-Laprade, P., and Petit, D., 2000 Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri* (Brassicaceae). *New Phytologist* **146:** 225-233.

Bert, V., Meerts, P., Saumitou-Laprade, P., Salis, P., Gruber, W., and Verbruggen, N., 2003 Genetic basis of Cd tolerance and hyperaccumulation in *Arabidopsis halleri*. *Plant and Soil* **249:** 9-18.

Boivin, K., Acarkan, A., Mbulu, R. S., Clarenz, O., and Schmidt, R., 2004 The Arabidopsis genome sequence as a tool for genome analysis in Brassicaceae. A comparison of the Arabidopsis and *Capsella rubella* genomes. *Plant Physiology* **135:** 735-744.

Courbot, M., Willems, G., Motte, P., Arfvidsson, S., Saumitou-Laprade, P., and Verbruggen, N., submitted to *Plant Journal* The major QTL for cadmium tolerance in *Arabidopsis halleri* co-localizes with *HMA4*, a gene encoding a Heavy Metal ATPase.

DeRisi, J. L., Iyer, V. R., and Brown, P. O., 1997 Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* **278:** 680-686.

Desbrosses-Fonrouge, A.-G., Voigt, K., Schröder, A., Arrivault, S., Thomine, S., and Krämer, U., 2005 *Arabidopsis thaliana* MTP1 is a Zn transporter in the vacuolar

membrane which mediates Zn detoxification and drives leaf Zn accumulation. *FEBS Letters* **579:** 4165-4174.

Diatchenko, L., Lau, Y.-F. C., Campbell, A. P., Chenchik, A., Moqadam, F., Huang, B., *et al.*, 1996 Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proceedings of the National Academy of Sciences of the U S A* **93:** 6025-6030.

Dräger, D. B., Desbrosses-Fonrouge, A.-G., Krach, C., Chardonnens, A. N., Meyer, R. C., Saumitou-Laprade, P., *et al.*, 2004 Two genes encoding *Arabidopsis halleri* MTP1 metal transport proteins co-segregate with zinc tolerance and account for high *MTP1* transcript levels. *Plant Journal* **39:** 425-439.

Eaves, I. A., Wicker, L. S., Ghandour, G., Lyons, P. A., Peterson, L. B., Todd, J. A., *et al.*, 2002 Combining mouse congenic strains and microarray gene expression analyses to study a complex trait: the NOD model of type 1 diabetes. *Genome Research* **12:** 232-243.

Feder, M. E., and Walser, J.-C., 2005 The biological limitations of transcriptomics in elucidating stress and stress responses. *Journal of Evolutionary Biology* **18:** 901-910.

Gracey, A. Y., and Cossins, A. R., 2003 Application of microarray technology in environmental and comparative physiology. *Annual Review of Physiology* **65:** 231-259.

Gracey, A. Y., Fraser, E. J., Li, W., Fang, Y., Taylor, R. R., Rogers, J., *et al.*, 2004 Coping with cold: an integrative, multitissue analysis of the transcriptome of a poikilothermic vertebrate. *Proceedings of the National Academy of Sciences of the U S A* **101:** 16970-16975.

Grotz, N., Fox, T., Connolly, E., Park, W., Guerinot, M. L., and Eide, D., 1998 Identification of a family of zinc transporter genes from Arabidopsis that respond to zinc deficiency. *Proceedings of the National Academy of Sciences of the U S A* **95:** 7220-7224.

Guerinot, M. L., and Eide, D., 1999 Zeroing in on zinc uptake in yeast and plants. *Current Opinion in Plant Biology* **2:** 244-249.

Guerinot, M. L., 2000 The ZIP family of metal transporters. *Biochimica et Biophysica Acta* **1465:** 190-198.

Hall, J. L., and Williams, L. E., 2003 Transition metal transporters in plants. *Journal of Experimental Botany* **54:** 2601-2613.

Hartley-Whitaker, J., Ainsworth, G., Vooijs, R., Ten Bookum, W., Schat, H., and Meharg, A. A., 2001 Phytochelatins are involved in differential arsenate tolerance in *Holcus lanatus*. *Plant Physiology* **126:** 299-306.

Krämer, U., 2005 MTP1 mops up excess zinc in *Arabidopsis* cells. *Trends in Plant Science* **10:** 313-315.

Kuittinen, H., de Haan, A. A., Vogl, C., Oikarinen, S., Leppala, J., Koch, M., *et al.*, 2004 Comparing the linkage maps of the close relatives *Arabidopsis lyrata* and *A. thaliana*. *Genetics* **168:** 1575-1584.

Lasat, M. M., Pence, N. S., Garvin, D. F., Ebbs, S. D., and Kochian, L. V., 2000 Molecular physiology of zinc transport in the Zn hyperaccumulator *Thlaspi caerulescens*. *Journal of Experimental Botany* **51:** 71-79.

Liu, H.-C., Cheng, H. H., Tirunagaru, V., Sofer, L., and Burnside, J., 2001 A strategy to identify positional candidated genes conferring Marek's disease resistance by integrating DNA microarrays and genetic mapping. *Animal Genetics* **32:** 351-359.

Macnair, M. R., Bert, V., Huitson, S. B., Saumitou-Laprade, P., and Petit, D., 1999 Zinc tolerance and hyperaccumulation are genetically independent characters. *Proceedings of the Royal Society of London, Series B: Biological Sciences* **266:** 2175-2179.

Mitchell-Olds, T., 2001 *Arabidopsis thaliana* and its wild relatives: a model system for ecology and evolution. *Trends in Ecology and Evolution* **16:** 693-700.

Munir, S., Singh, S., Kaur, K., and Kapur, V., 2004 Suppression subtractive hybridization coupled with microarray analysis to examine differential expression of genes in virus infected cells. *Biological Procedures Online* **6:** 94-104.

Murphy, A., and Taiz, L., 1995 Comparison of Metallothionein gene expression and nonprotein thiols in ten Arabidopsis ecotypes. *Plant Physiology* **109:** 945-954.

Pauwels, M., Saumitou-Laprade, P., Holl, A., Petit, D., and Bonnin, I., 2005 Multiple origin of metallicolous populations of the pseudometallophyte *Arabidopsis halleri* (Brassicaceae) in central Europe: the cpDNA testimony. *Molecular Ecology* **14:** 4403-4414.

Pence, N. S., Larsen, P. B., Ebbs, S. D., Letham, D. L., Lasat, M. M., Garvin, D. F., *et al.*, 2000 The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. *Proceedings of the National Academy of Sciences of the U S A* **97:** 4956-4960.

General discussion

Sagerström, C. G., Sun, B. I., and Sive, H. L., 1997 Subtractive cloning: Past, Present and Future. *Annual Review of Biochemistry* **66:** 751-783.

Schat, H., and Ten Bookum, W. M., 1992 Genetic control of copper tolerance in *Silene vulgaris*. *Heredity* **68:** 219-229.

van der Zaal, B. J., Neuteboom, L. W., Pinas, J. E., Chardonnens, A. N., Schat, H., Verkleij, J. A. C., *et al.*, 1999 Overexpression of a novel Arabidopsis gene related to putative zinc-transporter genes from animals can lead to enhanced zinc resistance and accumulation. *Plant Physiology* **119:** 1047-1055.

van Hoof, N. A. L. M., Hassinen, V. H., Hakvoort, H. W. J., Ballintijn, K. F., Schat, H., Verkleij, J. A. C., *et al.*, 2001 Enhanced copper tolerance in *Silene vulgaris* (Moench) Garcke populations from copper mines is associated with increased transcript levels of a 2b-type metallothionein gene. *Plant Physiology* **126:** 1519-1526.

Voorrips, R. E., 2002 Mapchart: Software for the graphical presentation of linkage maps and QTLs. *Heredity* **93:** 77-78.

Wayne, M. L., and McIntyre, L. M., 2002 Combining mapping and arraying: An approach to candidate gene identification. *Proceedings of the National Academy of Sciences of the USA* **99:** 14903-14906.

Weber, M., Harada, E., Vess, C., Roepenack-Lahaye, E., and Clemens, S., 2004 Comparative microarray analysis of *Arabidopsis thaliana* and *Arabidopsis halleri* roots identifies nicotianamine synthase, a ZIP transporter and other genes as potential metal hyperaccumulation factors. *Plant Journal* **37:** 269-281.

Willems, G., Godé, C., Dräger, D., Courbot, M., Verbruggen, N., and Saumitou-Laprade, P., submitted to *Genetics* Quantitative Trait Loci mapping of zinc tolerance in the metallophyte *Arabidopsis halleri* ssp. *halleri*.

Yogeeswaran, K., Frary, A., York, T. L., Amenta, A., Lesser, A. H., Nasrallah, J. B., *et al.*, 2005 Comparative genome analyses of *Arabidopsis* spp.: Inferring chromosomal rearrangement events in the evolutionary history of *A. thaliana*. *Genome Research* **15:** 505-515.

Zhou, J., and Goldsbrough, P. B., 1995 Structure, organization and expression of the metallothionein gene family in Arabidopsis. *Molecular Genetics and Genomics* **248:** 318-328.

7 GENERAL DISCUSSION

The present work contributes to the achievement of a better knowledge of the genetic and physiological bases underlying heavy metal tolerance in *A. halleri*. Although these issues have been previously addressed in numerous studies, a Quantitative Trait Loci (QTL) analysis had, until recently never been applied on metal tolerant species.

7.1 Heavy metal tolerance in the metallophyte *Arabidopsis halleri*

7.1.1 Genetic architecture of zinc and cadmium tolerance in A. halleri

Three genomic regions underlying Zn tolerance were identified in the QTL analysis performed on an interspecific *A. halleri* x *A. l. petraea* BC1 progeny. These three QTLs accounted together for 29% of the phenotypic variance or 42% of the genetic variance observed for this trait in the mapping population. As expected for *A. halleri* individuals growing on metalliferous sites the *A. halleri* allele at all three QTLs increased Zn tolerance. Epistatic interactions between the QTLs were significant, even though at a low significance threshold ($\alpha = 0.05$). Consequently, we might suppose that the QTLs have a predominantly additive effect. Metallicolous *A. halleri* populations were reported to exhibit a higher Zn tolerance under controlled conditions than most of the *A. halleri* populations growing on non-metalliferous soils (Bert *et al.* 2000; Pauwels *et al.* 2005). The same observation was previously done for metallicolous *T. caerulescens* populations when compared to non-metallicolous ones (Meerts and Van Isacker 1997). The authors suggested that "adding to the background level of constitutional tolerance specific tolerance mechanisms may have evolved in

metallicolous populations through natural selection in response to the local degree of heavy metal contamination of the soil" (Meerts and Van Isacker 1997). The metallicolous origin of the *A. halleri* parental genotype that was used for the generation of the BC1 progeny renders it impossible to distinguish among the QTLs, that we identified in this analysis, those that account for the constitutive Zn tolerance level from those that are involved in the quantitative variations observed at the intraspecific level. It might well be that the same genes are involved in both populations and that the increase in Zn tolerance in metallicolous populations relies on a different genetic regulation, leading to increased Zn tolerance levels in the latter. However, this needs to be verified for instance by a QTL analysis performed on intraspecific mapping populations developed on metallicolous and non-metallicolous *A. halleri* individuals or on interspecific *A. halleri* x *A. l. petraea* progenies using a non-metallicolous *A. halleri* individual.

Similarly to Zn tolerance, three QTLs conferring Cd tolerance were identified on a subset of the BC1 progeny, explaining up to 91% of the genetic variance of Cd tolerance in the BC1 progeny. Additionally, almost one half of the genetic variance (42%) was explained by a single QTL region, i.e. Cdtol-1. Contrary to Zn tolerance, we might thus presume a major gene (or rather QTL) to be present in the genetic determinism of Cd tolerance in *A. halleri*. Similarly to Zn tolerance, the *A. halleri* alleles at all three QTLs increased Cd tolerance of the BC1 genotypes. We assume that the QTLs have a predominant additive effect, because only one epistatic interaction at a significance threshold of 0.05 was detected.

Among the QTLs identified for Zn and Cd tolerance in the BC1 progeny, one QTL was apparently involved in both adaptive traits as inferred from the co-localisation of the QTLs Zntol-1 and Cdtol-1 on the genetic linkage map. This indicates the existence of partially independent genetic mechanisms conferring Zn and Cd tolerance in *A. halleri*. We suggest that the high Zn and Cd concentrations provoked unique adaptations, which are strongly, although not completely metal-specific, as previously reported for Cd and Zn tolerance in *S. vulgaris* populations (Schat *et al.* 1996; Schat and Vooijs 1997).

7.1.2 Physiological bases of zinc and cadmium tolerance in A. halleri

The resolution with which the QTLs were detected in our work (LOD support intervals > 4 cM) is insufficient to infer with certainty which genes are involved in either Zn or Cd tolerance in *A. halleri*. Nevertheless, the mapping of seven metal homeostasis genes on the *A. halleri* x *A. l. petraea* genetic linkage map enabled us to propose potential candidate genes for both traits.

The QTLs For Zn tolerance mapped to the three metal homeostasis genes *HMA4, MTP1-A* and *MTP1-B*. An extensive functional characterisation of the metal transporter gene *MTP1* with regard to its role in Zn homeostasis was performed in *A. thaliana* (Kobae *et al.* 2004; Desbrosses-Fonrouge *et al.* 2005; Krämer 2005) as well as in *A. halleri* (Dräger *et al.* 2004). The expression of *MTP1* in yeast cells, the analysis of *A. thaliana* mutant lines carrying a non-functional MTP1 or overexpressing the gene and finally, the cellular localisation of MTP1 in the vacuolar membrane supported a role for MTP1 in Zn detoxification in *A. thaliana* and *A. halleri* through the vacuolar sequestration of the metal (Dräger *et al.* 2004; Kobae *et al.* 2004; Desbrosses-Fonrouge *et al.* 2005). With regard to the expression and regulation of *MTP1* in *A. halleri* Dräger *et al.* (2004) suggested that "at low Zn concentrations relatively low rates of Zn sequestration in the vacuoles of the root cells of *A. halleri* may enable the transport of Zn from the roots to the shoots, thus permitting metal hyperaccumulation in the shoot. At high Zn supply, enhanced Zn sequestration in the root cells of *A. halleri* may participate in protecting the shoot from accumulating excess Zn." The metal pump HMA4, a member of the P_{1B}-type ATPases was studied with regard to its role in Cd and Zn homeostasis in *A. thaliana* (Hussain *et al.* 2004; Verret *et al.* 2004; Mills *et al.* 2005; Verret *et al.* 2005), in the metal tolerant and hyperaccumulator species *T. caerulescens* (Bernard *et al.* 2004; Papoyan and Kochian 2004) and recently, in *A. halleri* (Courbot *et al.* submitted). At the cellular level a role for HMA4 in Zn and Cd homeostasis through the efflux of these metals was suggested for *A. thaliana* as well as for the metallophyte species (Mills *et al.* 2003; Hussain *et al.* 2004). In the plant AtHMA4 was supposed to contribute to the root to shoot translocation of Zn and Cd (Verret *et al.* 2004). The

predominant expression of *HMA4* in the roots of *T. caerulescens* and *A. halleri* plants supports a role for TcHMA4 and AhHMA4 in root to shoot translocation as suggested for AtHMA4 (Bernard *et al.* 2004; Papoyan and Kochian 2004; Courbot *et al.* submitted).

In the light of our work, in which we quantified Zn tolerance more precisely by evaluating root growth, a role for MTP1 and HMA4 in Zn tolerance might be expected through the protection of the root cells against the accumulation of high cytosolic Zn concentrations. This might be accomplished through the sequestration of Zn in the vacuole by MTP1 and the export of Zn by HMA4 (Figure 7.1).

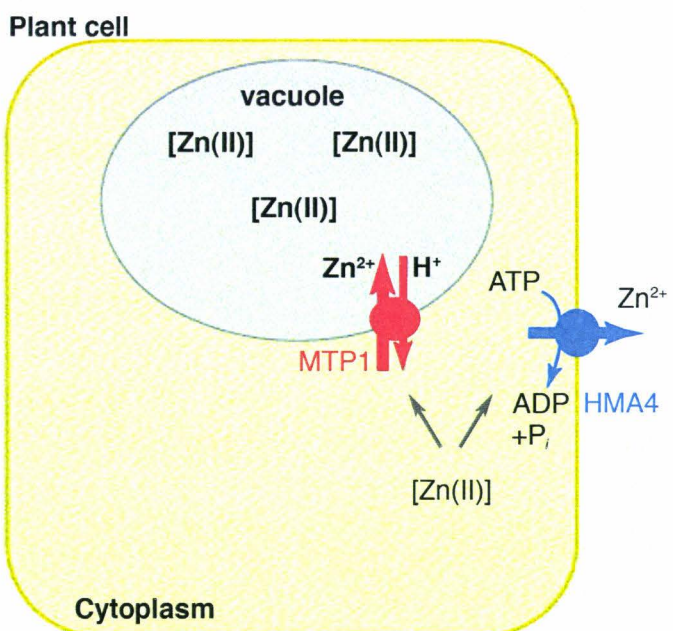


Figure 7.1 Mechanisms conferring Zn tolerance in *A. halleri* root cells as indicated by the QTL analysis, according to Krämer (2005).

The major QTL for Cd tolerance co-localised with the metal homeostasis gene *HMA4*. Similarly to the role of HMA4 in Zn tolerance, HMA4 might be expected to contribute to Cd tolerance in the *A. halleri* root cells through the transport of Cd out of the cytoplasm. Genes that might play a role in metal homeostasis were not mapped within the remaining QTL regions, Cdtol-2 and Cdtol-3. Moreover, the LOD support intervals associated with these QTLs were of considerable length, which precluded any

suggestion of potential candidate genes for instance on the basis of the *A. thaliana* genome sequence. As for Zn tolerance vacuolar compartmentalisation of Cd is believed to be involved in Cd tolerance (Sanito di Toppi and Gabbrielli 1999). However, to date transporters of Cd that might be responsible for the vacuolar compartmentalisation of this metal have not been identified yet.

7.1.3 Evolution of heavy metal tolerance in A. halleri

Although the origin of heavy metal tolerance in the *A. halleri* species has not been dated exactly, it is clear that this trait appeared long before the occurrence of metal contaminated sites due to industrial activities given the constitutional presence of this trait in *A. halleri*. Thus, heavy metal tolerance might have appeared up to 2.9 million years ago in *A. halleri*, i.e. the divergence time between *A. halleri* and its closest relative, the *A. lyrata* species. The QTL mapping analyses on Zn and Cd tolerance indicated the existence of a common genomic region in both Zn and Cd tolerance; this QTL was the major QTL conferring Cd tolerance in *A. halleri*.

Metallicolous *A. halleri* populations occur mainly on calamine soils, which are characterised by high Zn, Cd and Pb contaminations (Bert *et al.* 2002). Sites naturally enriched in these heavy metals have been reported in the Harz mountains (Germany) and Silesia (Poland) (Bert *et al.* 2002; Pauwels *et al.* 2005). Heavy metal tolerance is believed to have appeared on these naturally contaminated sites where ore bodies must have been exposed at the surface of the ground long before there was any mining (Bradshaw 1970; Ernst 1974). Based on our findings we might suggest that the evolution of heavy metal tolerance in *A. halleri* was initiated through the fixation of the QTL common to Zn and Cd tolerance, since this should have enabled non-tolerant *A. halleri* individuals to colonise sites polluted by both metals. Increased tolerance to Zn and Cd might then have been achieved through the acquisition of mechanisms specific to either Zn or Cd tolerance. The evolutionary history we suggest for heavy metal tolerance in the *A. halleri* species is consistent with the exponential model of adaptation established by Orr (1998), in which he stated that the first factors fixed can be fairly large. Ultimately however, it should be verified if the QTL region identified in our

study to confer both Zn and Cd tolerance in a metallicolous *A. halleri* individual is also involved in Zn and Cd tolerance in *A. halleri* individuals growing on non-metallicolous sites.

7.2 Zinc hyperaccumulation in the metallophyte *Arabidopsis halleri*

Similarly to Zn and Cd tolerance the hyperaccumulation of these metals was reported to be a constitutive property of the *A. halleri* species (Macnair *et al.* 1999; Bert *et al.* 2000; Bert *et al.* 2002). However, variations for Zn and Cd hyperaccumulation were observed between and within populations under field (Bert *et al.* 2000; Bert *et al.* 2002) and controlled conditions (Macnair 2002). Through the evaluation under controlled conditions of the Zn hyperaccumulation ability of six F1 populations, generated on crosses between either a metallicolous (three crosses) or a non-metallicolous (three crosses) *A. halleri* individual and an *A. l. petraea* individual, we were able to confirm the high level of variability previously reported for Zn hyperaccumulation in the *A. halleri* species (data not shown).

In contrast to the results reported by Macnair *et al.* (1999) we could not confirm the dominant character of the hyperaccumulation property in *A. halleri* (Macnair *et al.* 1999; Macnair *et al.* 2000), since high foliar Zn concentrations were found neither in the F1 parental genotype nor in the *A. halleri* x *A. l. petraea* BC1 individuals upon growth on non-polluted and artificially contaminated soil. However, the metal transporters HMA4 and MTP1 that co-localised with the QTLs conferring Zn tolerance, were suggested to be involved in root to shoot translocation (HMA4) (Bernard *et al.* 2004; Verret *et al.* 2004; Courbot *et al.* submitted) and foliar Zn sequestration (MTP1) (Dräger *et al.* 2004) upon their functional analysis (Figure 7.3).

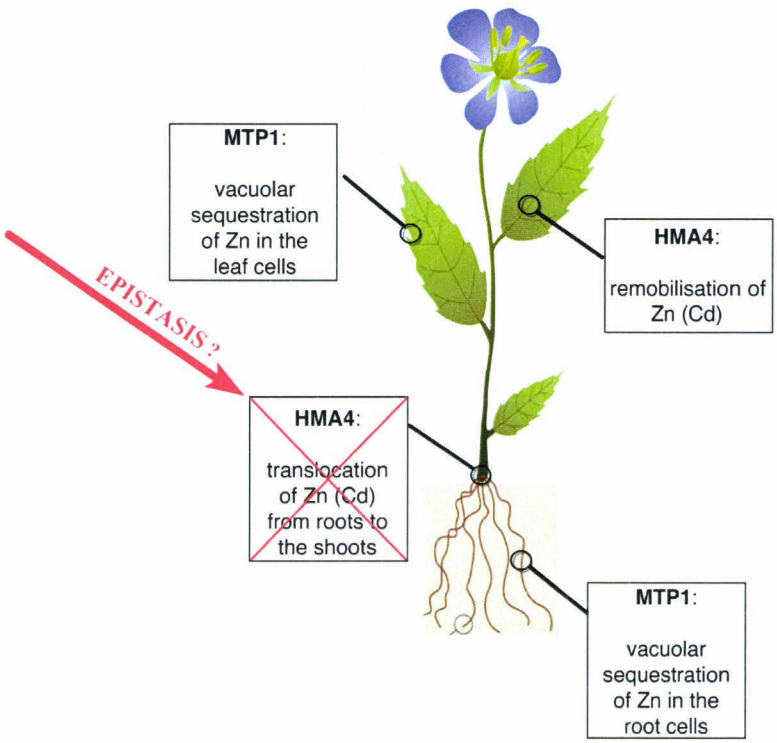


Figure 7.3 Contribution of *HMA4* and *MTP1* to Zn hyperaccumulation in *A. halleri*, according to Clemens *et al.* 2001

In the *A. halleri* x *A. l. petraea* BC1 progeny Zn hyperaccumulation has not been observed. Through epistasis, acting on *HMA4* and, preventing the translocation of Zn to the shoots the absence of Zn hyperaccumulation in the BC1 might be explained.

Thus, the absence of Zn hyperaccumulation in the *A. halleri* x *A. l. petraea* BC1 progeny is rather surprising. Apparently, translocation and leaf sequestration capacities are not sufficient to accumulate high amounts of Zn in the aboveground parts and one or more other mechanisms seem to be involved in this trait. Increased metal uptake and metal foraging capacities for instance may significantly contribute to the ability of concentrating high Zn amounts in the leaves. Otherwise, we might explain the lack of Zn hyperaccumulation in the *A. halleri* x *A. l. petraea* BC1 progeny through the existence of a gene that interacts epistatically with the metal pump HMA4 and prevents Zn from being translocated to the shoots (Figure 7.3).

Although the absence of Zn hyperaccumulation in the BC1 progeny precluded the identification of the QTLs involved in this trait we were able to infer an interesting aspect regarding Zn hyperaccumulation through the comparative analysis of non-

hyperaccumulating and hyperaccumulating individuals (G. Sarret, unpublished results). The analysis of *A. halleri* and *A. lyrata* leaves by μXRF revealed a distinct distribution pattern of Zn within the leaves of both species. Whereas in the *A. halleri* leaves the inner leaf tissue was highly enriched in Zn ($Zn_{vein}/Zn_{tissue} < 1$), in *A. lyrata* leaves the metal remained mostly in the veins ($Zn_{vein}/Zn_{tissue} > 1$) (Figure 7.2B).

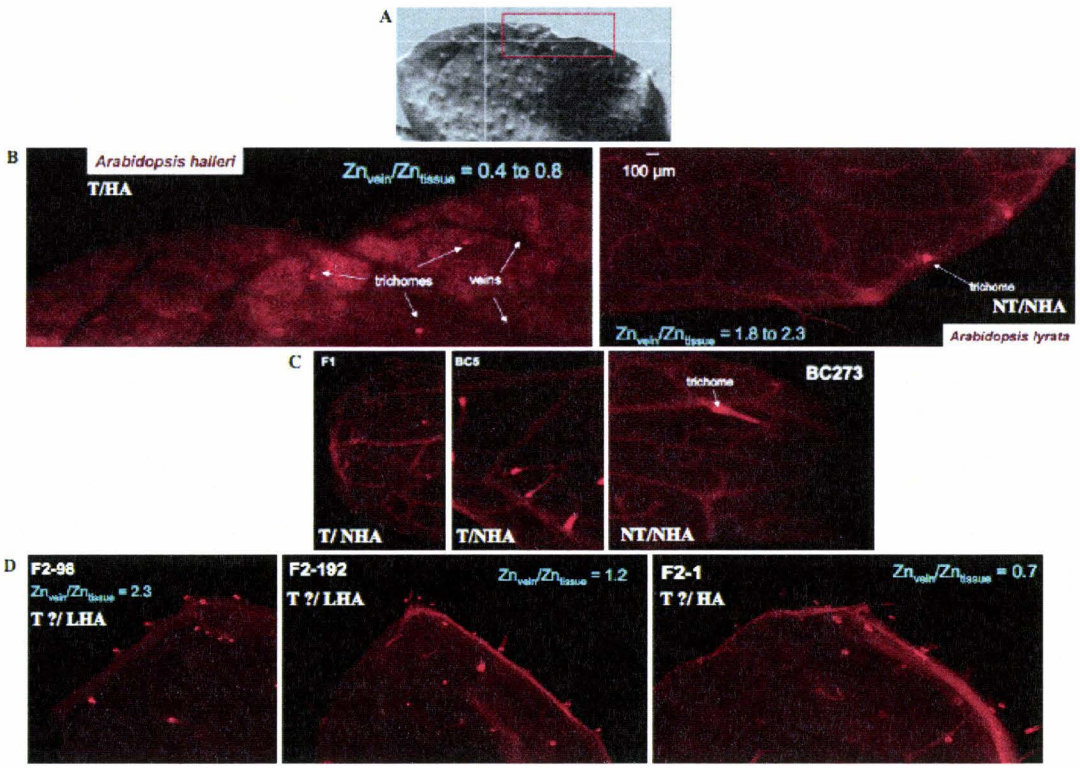


FIGURE 7.2 Distribution pattern of Zn in the leaves of hyperaccumulating and non-hyperaccumulating individuals (G. Sarret, unpublished results).

A: image of *A. halleri* leaf in red the part of the leaf on which XRF analysis has been performed; B: *A. halleri* (left panel), *A. l. petraea* (right panel); C: F1 (left panel), BC1 individuals (middle and right panel; D: F2 individuals (left, middle and right panel). T tolerant, NT non-tolerant, HA hyperaccumulating, NHA non-hyperaccumulating, LHA lowly hyperaccumulating.

The subsequent analysis of the F1 parental line of the BC1 progeny as well as of four BC1 individuals with contrasting Zn tolerance phenotypes showed that this distribution pattern of Zn was specific to the *A. halleri* species. Independently of their Zn tolerance level, all individuals exhibited a Zn distribution pattern within the leaves similar to *A. lyrata* (Figure 7.2C). Interestingly, the analysis of four *A. halleri* x *A. l.*

petraea F2 individuals[7] with contrasting Zn hyperaccumulation phenotypes revealed a Zn distribution pattern similar to the one observed in *A. halleri* ($Zn_{vein}/Zn_{tissue} < 1$) only in the F2 individual exhibiting a high Zn accumulation capacity comparable to *A. halleri* (Figure 7.2D). These findings indicate that the storage of Zn in the inner leaf tissue plays an essential role in Zn hyperaccumulation, rather than in Zn tolerance.

[7] These individuals originated from a newly developed F2 progeny generated on two independent crosses between a metallicolous *A. halleri* individual and an *A. l. petraea* individual (Saumitou-Laprade, P.). This F2 progeny segregates for Zn hyperaccumulation as observed upon growth on non-polluted or artificially polluted soil under controlled conditions.

7.3 References

Bernard, C., Roosens, N., Czernic, P., Lebrun, M., and Verbruggen, N., 2004 A novel CPx-ATPase from the cadmium hyperaccumulator *Thlaspi caerulescens*. *FEBS Letters* **569:** 140-148.

Bert, V., Macnair, M. R., de Laguerie, P., Saumitou-Laprade, P., and Petit, D., 2000 Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri* (Brassicaceae). *New Phytologist* **146:** 225-233.

Bert, V., Bonnin, I., Saumitou-Laprade, P., de Laguerie, P., and Petit, D., 2002 Do *Arabidopsis halleri* from nonmetallicolous populations accumulate zinc and cadmium more effectively than those from metallicolous populations? *New Phytologist* **155:** 47-57.

Bradshaw, A. D., 1970 Plants and industrial waste. *Transactions of the Botanical Society of Edinburgh* **41:** 71-84.

Courbot, M., Willems, G., Motte, P., Arfvidsson, S., Saumitou-Laprade, P., and Verbruggen, N., submitted to *Plant Journal* The major QTL for cadmium tolerance in *Arabidopsis halleri* co-localizes with *HMA4*, a gene encoding a Heavy Metal ATPase.

Desbrosses-Fonrouge, A.-G., Voigt, K., Schröder, A., Arrivault, S., Thomine, S., and Krämer, U., 2005 *Arabidopsis thaliana* MTP1 is a Zn transporter in the vacuolar membrane which mediates Zn detoxification and drives leaf Zn accumulation. *FEBS Letters* **579:** 4165-4174.

Dräger, D. B., Desbrosses-Fonrouge, A.-G., Krach, C., Chardonnens, A. N., Meyer, R. C., Saumitou-Laprade, P., *et al.*, 2004 Two genes encoding *Arabidopsis halleri* MTP1 metal transport proteins co-segregate with zinc tolerance and account for high *MTP1* transcript levels. *Plant Journal* **39:** 425-439.

Ernst, W. H. O., 1974 *Schwermetallvegetation der Erde*. G. Fischer, Stuttgart, Germany.

Hussain, D., Haydon, M. J., Wang, Y., Wong, E., Sherson, S. M., Young, J., *et al.*, 2004 P-Type ATPase Heavy Metal Transporters with roles in essential zinc homeostasis in Arabidopsis. *Plant Cell* **16:** 1327-1339.

Kobae, Y., Uemura, T., Sato, M. H., Ohnishi, M., Mimura, T., Nakagawa, T., *et al.*, 2004 Zinc transporter of *Arabidopsis thaliana* AtMTP1 is localized to vacuolar membranes and implicated in zinc homeostasis. *Plant and Cell Physiology* **45:** 1749-1758.

Krämer, U., 2005 MTP1 mops up excess zinc in *Arabidopsis* cells. *Trends in Plant Science* **10:** 313-315.

Macnair, M. R., Bert, V., Huitson, S. B., Saumitou-Laprade, P., and Petit, D., 1999 Zinc tolerance and hyperaccumulation are genetically independent characters. *Proceedings of the Royal Society of London, Series B: Biological Sciences* **266:** 2175-2179.

Macnair, M. R., Tilstone, G. H., and Smith, S. E., 2000 The genetics of metal tolerance and accumulation in higher plants, pp. 235-250 in *Phytoremediation of contaminated soil and water*, edited by C. Press.

Macnair, M. R., 2002 Within and between population genetic variation for zinc accumulation in *Arabidopsis halleri*. *New Phytologist* **155:** 59-66.

Meerts, P., and Van Isacker, N., 1997 Heavy metal tolerance and accumulation in metallicolous and non-metallicolous populations of *Thlaspi caerulescens* from continental Europe. *Plant Ecology* **133:** 221-231.

Mills, R., Krijger, G., Baccarini, P., Hall, J. L., and Williams, L., 2003 Functional expression of AtHMA4, a P_{1B}-type ATPase of the Zn/Co/Cd/Pb subclass. *Plant Journal* **35:** 164-176.

Mills, R. F., Francini, A., Ferreira da Rocha, P. S., Baccarini, P. J., Aylett, M., Krijger, G. C., *et al.*, 2005 The plant P1B-type ATPase AtHMA4 transports Zn and Cd and plays a role in detoxification of transition metals supplied at elevated levels. *FEBS Letters* **579:** 783-791.

Orr, H. A., 1998 The population genetics of adaptation: the distribution of factors fixed during adaptive evolution. *Evolution* **52:** 935-949.

Papoyan, A., and Kochian, L. V., 2004 Identification of *Thlaspi caerulescens* genes that may be involved in heavy metal hyperaccumulation and tolerance. Characterization of a novel heavy metal transporting ATPase. *Plant Physiology* **136:** 3814-3823.

Pauwels, M., Saumitou-Laprade, P., Holl, A., Petit, D., and Bonnin, I., 2005 Multiple origin of metallicolous populations of the pseudometallophyte *Arabidopsis*

Perspectives

halleri (Brassicaceae) in central Europe: the cpDNA testimony. *Molecular Ecology* **14:** 4403-4414.

Sanito di Toppi, L., and Gabbrielli, R., 1999 Response to cadmium in higher plants. *Environmental and Experimental Botany* **41:** 105-130.

Schat, H., Vooijs, R., and Kuiper, E., 1996 Identical major gene loci for heavy metal tolerances that have independently evolved in different local populations and subspecies of *Silene vulgaris*. *Evolution* **50:** 1888-1895.

Schat, H., and Vooijs, R., 1997 Multiple tolerance and co-tolerance to heavy metals in *Silene vulgaris*: a co-segregation analysis. *New Phytologist* **136:** 489-496.

Verret, F., Gravot, A., Auroy, P., Leonhardt, N., David, P., Nussaume, L., *et al.*, 2004 Overexpression of AtHMA4 enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. *FEBS Letters* **576:** 306-312.

Verret, F., Gravot, A., Auroy, P., Preveral, S., Forestier, C., Vavasseur, A., *et al.*, 2005 Heavy metal transport by AtHMA4 involves the N-terminal degenerated metal binding domain and the C-terminal His11 stretch. *FEBS Letters* **579:** 1515-1522.

8 PERSPECTIVES

In line with the results obtained in the present work, we propose several research topics that may contribute to a better comprehension of heavy metal tolerance and hyperaccumulation in *A. halleri*:

- **A fine-mapping analysis of the QTLs conferring Zn and Cd tolerance:** this is essential either to identify the genes involved in these traits or to confirm the implication in Zn/Cd tolerance of the candidate genes that we proposed in the present work. In this aim we will need to increase the resolution with which the QTLs have been positioned on the *A. halleri* x *A. l. petraea* linkage map. At first, this can be achieved by the genotyping of the BC1 progeny with additional molecular markers. We have several resources allowing us to target specifically the QTL regions that we identified in the present work:

(1) The *A. thaliana* genome sequence, because of the extensive macrosynteny that we observed between the *A. halleri* x *A. l. petraea* and the *A. thaliana* map;

(2) The differentially expressed genes identified through transcriptome analyses that co-localised with the QTL regions of Zn tolerance. A similar approach as we applied in chapter 6 on Zn tolerance should be conducted to identify positional candidate genes for Cd tolerance. Coding sequences differentially expressed in Cd-tolerant BC1 individuals compared to Cd-sensitive ones have been isolated through a cDNA-AFLP analysis (N. Verbruggen, unpublished results) and could be used in this purpose;

(3) A bacterial artificial chromosome (BAC) library, created on the *A. halleri* parental genotype of the BC1 progeny (M. Lebrun, unpublished results). The use of this information resource might be successful for the design of markers specific to the QTL region Zntol-2, which mapped to the end of the linkage group LG4. In order to delimit this QTL region, we should position markers beyond the marker *MTP1-A*. Since this

marker mapped to the lower extremity of the second *A. thaliana* chromosome, the use of the BAC clones carrying this QTL region might facilitate the development of markers specific to this region. In addition, the BAC library might also be a useful tool for the mapping of those regions that have been lost in the *A. thaliana* genome in the transition from eight to five chromosomes. In *A. halleri* for instance three copies were found of the *AtMTP1* ortholog, whereas only one copy of this gene was present in *A. thaliana* (Dräger *et al.* 2004). A lower genome size was moreover reported for *A. thaliana* (~ 0.16 pg) compared to *A. halleri* and *A. lyrata* (~ 0.26 pg) (Dart *et al.* 2004; Johnstone *et al.* 2005); other duplication events might consequently be identified in *A. halleri* as well as in the other wild Arabidopsis relatives.

Since the number of recombination events that took place in the generation of the BC1 progeny is limited (only one meiosis), the increase in resolution obtained by the mapping of additional markers will also be limited. In order to obtain a significant increase in resolution, we should consider the production of progenies generated by repeated backcrossing on the recurrent parental line (*A. l. petraea*). In this aim we can use BC1 individuals carrying either all QTL regions or only one of the QTL regions implied in the metal tolerance traits. The segregation of only one QTL region in the progeny will allow us to confirm the significant contribution of the QTL region to the trait and to characterise each QTL individually, which will provide us a more accurate estimate of its effect and contribution to the trait. It is noteworthy however, that high-resolution mapping can be very time-consuming, especially when dealing with non-model species that require a much longer generation time than the currently used model species. The realisation of a fine-mapping analysis implies also high genotyping costs, because of the high number of markers to map and the large population sizes that are required. Moreover, the evaluation of metal tolerance might imply high costs. The feasibility of applying such a strategy should consequently be verified in our experiment.

- **The study of the relationship between Zn/Cd tolerance and Zn/Cd hyperaccumulation:** the relationship between these traits has already been investigated in *A. halleri* (Macnair *et al.* 1999; Bert *et al.* 2003) as well as in *T. caerulescens*

(Assunçao *et al.* 2003; Frerot *et al.* 2005). However, these studies were limited to the analysis of the co-segregation of both traits in inter- or intraspecific progenies and remained consequently rather descriptive. Through the application of a QTL mapping approach we will be able to analyse at first the co-localisation of the QTLs involved in metal tolerance and hyperaccumulation, which will inform us on the correlation between both traits (do the QTLs co-localise?) and, if such a correlation exists, to which extent both traits are correlated (how many QTLs do co-localise?). In this aim an *A. halleri* x *A. l. petraea* F2 progeny has been generated (P. Saumitou-Laprade, unpublished results) on which the QTL mapping of Zn hyperaccumulation is currently in progress. The QTL analysis of Zn tolerance on this mapping population will be shortly initiated, allowing the investigation of the relationship between both Zn tolerance and hyperaccumulation in *A. halleri*. Moreover, the genetic linkage map of the F2 progeny is constructed through the use of molecular markers identical to those mapped on the *A. halleri* x *A. l. petraea* BC1 progeny. The co-localisation of the QTLs conferring Zn tolerance identified in the BC1 progeny and, hyperaccumulation identified in the F2 progeny will provide us a first insight in the relationship between both traits.

- **Genetic analysis of metal tolerance and hyperaccumulation at the intraspecific level:** In addition to the application of QTL analyses of metal tolerance and hyperaccumulation on interspecific *A. halleri* x *A. l. petraea* mapping populations, we should consider the use of intraspecific *A. halleri* mapping populations to identify the genes responsible for the quantitative variations observed within the *A. halleri* species between metallicolous and non-metallicolous populations. However, in order to investigate the genetic bases of the quantitative variations relative to Zn tolerance, an improved phenotyping method needs to be developed. The sequential exposure test used in the present work might reveal not sensitive enough for addressing the QTL analysis of Zn tolerance at the intraspecific level. Regarding Zn hyperaccumulation we developed a semi-automated, sensitive, reliable and low-cost phenotyping method, which should enable us to reveal even small differences in Zn accumulation capacities and to perform consequently, a QTL analysis of Zn accumulation on intraspecific mapping populations.

Annexes

References

Assunçao, A. G. L., Ten Bookum, W. M., Nelissen, H. J. M., Vooijs, R., Schat, H., and Ernst, W. H. O., 2003 A cosegregation analysis of zinc (Zn) accumulation and Zn tolerance in the Zn hyperaccumulator *Thlaspi caerulescens*. *New Phytologist* **159:** 383-390.

Bert, V., Meerts, P., Saumitou-Laprade, P., Salis, P., Gruber, W., and Verbruggen, N., 2003 Genetic basis of Cd tolerance and hyperaccumulation in *Arabidopsis halleri*. *Plant and Soil* **249:** 9-18.

Dart, S., Kron, P., and Mable, B. K., 2004 Characterizing polyploidy in *Arabidopsis lyrata* using chromosome counts and flow cytometry. *Canadian Journal of Botany* **82:** 185-197.

Dräger, D. B., Desbrosses-Fonrouge, A.-G., Krach, C., Chardonnens, A. N., Meyer, R. C., Saumitou-Laprade, P., *et al.*, 2004 Two genes encoding *Arabidopsis halleri* MTP1 metal transport proteins co-segregate with zinc tolerance and account for high *MTP1* transcript levels. *Plant Journal* **39:** 425-439.

Frerot, H., Lefebvre, C., Petit, C., Collin, C., Dos Santos, A., and Escarre, J., 2005 Zinc tolerance and hyperaccumulation in F1 and F2 offspring from intra and interecotype crosses of *Thlaspi caerulescens*. *New Phytologist* **165:** 111-119.

Johnstone, J. S., Pepper, A. E., Hall, A. E., Chen, Z. J., Hodnett, G., Drabek, J., *et al.*, 2005 Evolution of genome size in Brassicaceae. *Annals of Botany* **95:** 229-235.

Macnair, M. R., Bert, V., Huitson, S. B., Saumitou-Laprade, P., and Petit, D., 1999 Zinc tolerance and hyperaccumulation are genetically independent characters. *Proceedings of the Royal Society of London, Series B: Biological Sciences* **266:** 2175-2179.

9 ANNEXES

BOX 1: Definition and biological function of heavy metals.

The term "heavy metals" designates those elements having densities greater than 5 g/cm^3 (Antonovics *et al.* 1971). Heavy metals, such as copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn) are essential micronutrients for normal growth and development in eukaryotic organisms, which implies that they are required in the organism in trace amounts. Cu for instance is an integral component of electron transfer reactions mediated by proteins such as superoxide dismutase, cytochrome c oxidase and plastocyanin and is implicated in photosynthesis and respiration similarly to Mn. Zn has a key structural and/or catalytic role in many proteins and enzymes, such as carbonic anhydrase, alcohol dehydrogenase, superoxide dismutase, RNA polymerase and is also involved in nitrogen metabolism (Guerinot and Eide 1999; Hagemeyer 1999; Clemens 2001; Hall and Williams 2003). Whole genome data of the eukaryotic organisms *Saccharomyces cerevisae* and *Chlamydomonas elegans* revealed sequence motifs characteristic of zinc binding structural domains for more than 3% of the protein sequences (Guerinot and Eide 1999; Clemens 2001). In *Arabidopsis thaliana* Zn is an essential cofactor for more than 1200 transcription factors, protein interaction domains and enzymes (Krämer 2005). Other heavy metals, like cadmium (Cd), nickel (Ni), lead (Pb) and mercury (Hg) are nonessential and have to date no established function in the metabolism of eukaryotic organisms (Clemens 2001; Hall 2002). In relation to biological life, essential and nonessential heavy metals have a common feature in that they are severely damaging in excessive quantities. Heavy metal excess has been demonstrated to stimulate the formation of free radicals and reactive oxygen species either by direct electron transfer involving metal cations or as a consequence of metal-mediated inhibition of metabolic reactions (Dietz *et al.* 1999; Clemens 2001; Hall 2002). Free radicals and reactive oxygen species will in turn react with many cellular constituents, for example macromolecules such as proteins, nucleic acids and lipids (Dietz *et al.* 1999). Moreover, high heavy metal concentrations may lead to the inactivation or the disruption of the structure of proteins, because of their high affinity for sulphydryl and amino groups (Clemens 2001; Hall 2002).

References

Antonovics, J., Bradshaw, A. D., and Turner, R. G., 1971 Heavy metal tolerance in plants. *Advances in Ecological Research* **7:** 1-85.

Clemens, S., 2001 Molecular mechanisms of plant metal tolerance and homeostasis. *Planta* **212:** 475-486.

Dietz, K.-J., Baier, M., and Krämer, U., 1999 Free radicals and reactive oxygen species as mediators of heavy metal toxicity in plants in *Phytoremediation of heavy metal contaminated and polluted soils*, edited by M. N. V. Prasad and J. Hagemeyer. Springer, Berlin.

Guerinot, M. L., and Eide, D., 1999 Zeroing in on zinc uptake in yeast and plants. *Current Opinion in Plant Biology* **2:** 244-249.

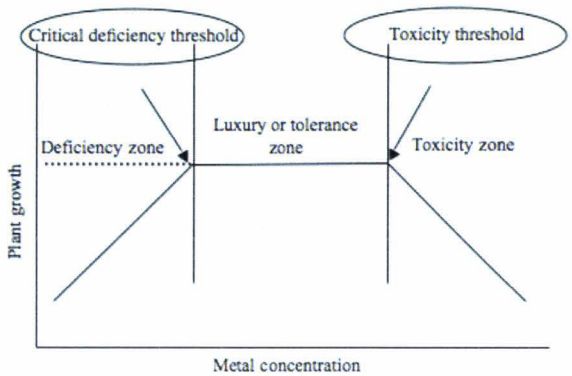
Hagemeyer, J., 1999 Ecophysiology of plant growth under heavy metal stress in *Phytoremediation of heavy metal contaminated and polluted soils*, edited by M. N. V. Prasad and J. Hagemeyer. Springer, Berlin.

Hall, J. L., 2002 Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of Experimental Botany* **53:** 1-11.

Hall, J. L., and Williams, L. E., 2003 Transition metal transporters in plants. *Journal of Experimental Botany* **54:** 2601-2613.

Krämer, U., 2005 MTP1 mops up excess zinc in *Arabidopsis* cells. *Trends in Plant Science* **10:** 313-315.

BOX 2: The dose-response curve, according to (Macnair 1993; Hagemeyer 1999)



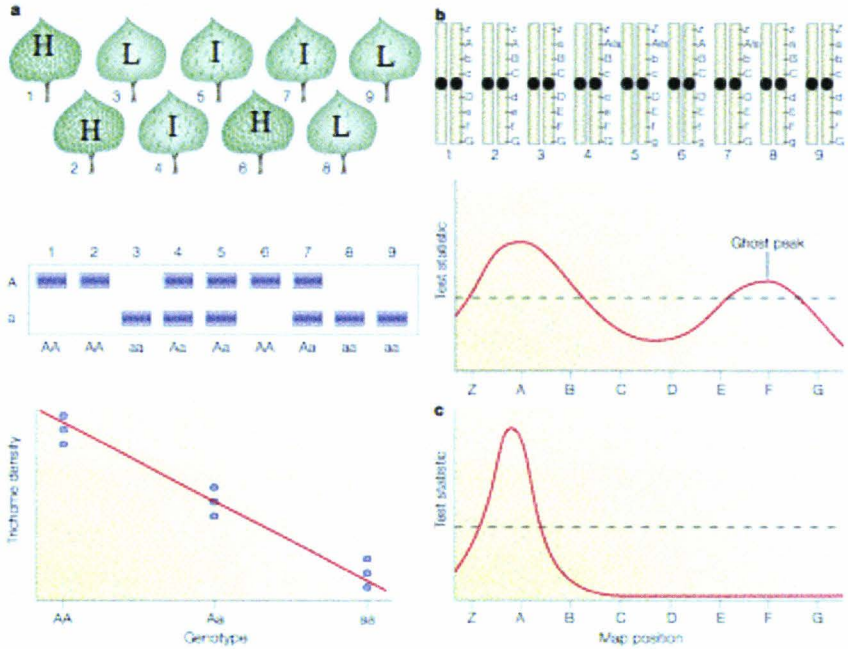
The dose-response-curve plotting plant growth in function of metal concentration is composed of three phases in the case of the essential heavy metals (full line on the graph): the deficiency phase, the luxury or tolerance phase and the toxicity phase. In the deficiency phase plants will show deficiency symptoms because of a lack of one or more essential elements. The plant growth will consequently be stimulated upon an increase of the concentration of these elements until the critical deficiency threshold, i.e. the concentration required for normal plant development, is exceeded. In the luxury phase plant growth will not be further stimulated in response to an increase of the metal concentration. Since plants will not exhibit any toxicity symptoms, indicating that they are able to cope with varying concentrations of heavy metals (although exceeding the critical deficiency threshold) the luxury phase is also called the tolerance phase. An additional increase in the concentration above the toxicity threshold, i.e. the highest concentration at which the plant can maintain homeostasis, will involve a severe inhibition of plant growth; these concentrations characterise the toxicity phase. Plants will not be stimulated upon an increase of the concentrations of nonessential elements (the dashed line on the graph), as observed for essential heavy metals below the deficiency threshold. Only two phases are therefore distinguished in the dose-response-curve established for nonessential elements: the luxury or tolerance phase and the toxicity phase. As observed for essential elements plants have the ability to tolerate varying concentrations of nonessential elements within a certain range, i.e. until the toxicity threshold is exceeded. Similarly as for high concentrations of essential nutrients plants will exhibit toxicity symptoms to concentrations exceeding the toxicity threshold.

References

Hagemeyer, J., 1999 Ecophysiology of plant growth under heavy metal stress in *Phytoremediation of heavy metal contaminated and polluted soils*, edited by M. N. V. Prasad and J. Hagemeyer. Springer, Berlin.

Macnair, M. R., 1993 Tansley review No. 49: The genetics of metal tolerance in vascular plants. *New Phytologist* **124:** 541-559.

BOX 3: Presentation of the different QTL mapping methods. The methods are illustrated by the example of trichome density in leaves, according to Mauricio *et al.* (2001).



(a) Single-marker analysis

Upper panel: Trichome density has been measured for 9 individuals of the mapping population (high (H), intermediate (I) and low (L) trichome density); *Middle panel:* Genotyping data of the individuals for the marker locus A following the migration of DNA fragments on the gel. The genotypes of each individual are indicated beneath the migration pattern. Three different genotypes can be distinguished (AA, Aa, aa); *Lower panel:* Trichome density is plotted in function of marker genotype. A significant relationship is identified between marker locus A and trichome density, which indicates that a QTL for trichome density is probably linked to the marker locus A.

(b) Interval mapping

Upper panel: A genetic linkage map is constructed on the mapping population; *Lower panel:* The test statistic (usually LOD score) corresponding to the probability of the presence of a QTL is calculated at different positions (for instance every 1 cM) between two flanking markers. The position at which the test statistic reaches its maximum, if exceeding the significance threshold (indicated by the dashed line) corresponds to the most likely position of the QTL. The presence of nearby QTLs can reduce the power of QTL detection in IM and can result in the detection of false or so-called ghost QTLs (the ghost peak on the graph).

(c) Multiple QTL models

Markers are added to the model to take over the role of the other QTLs when searching for a QTL. These methods provide a more accurate estimation of the QTL position than IM. A higher test statistic value will be obtained for the QTL peak and the QTL peak will be narrowed down (see graph) when compared to IM.

References

Mauricio, R., 2001 Mapping Quantitative Trait Loci in plants: uses and caveats for evolutionary biology. *Nature Reviews Genetics* **2:** 370-380.

TABLE 1 Zn tolerance EC100 values (μM Zn)

N° plante	EC100	N° plant	EC100	N° plant	EC100	N° plant	EC100	N° plant	EC100
1	75	34	100	72	25	99	250	133	150
2	25	34	250	72	50	99	1000	133	150
2	50	35	50	72	250	101	25	135	25
2	100	38	25	73	75	101	100	135	50
4	500	38	75	74	25	103	25	137	250
4	1000	40	500	74	100	103	50	137	500
5	2000	40	500	74	250	103	50	137	1000
5	3000	40	1000	75	1000	105	25	138	50
6	150	42	50	75	2000	105	75	138	50
7	50	42	50	76	75	106	150	141	50
7	250	44	50	76	1000	108	25	143	250
9	500	44	100	77	25	108	50	144	1000
11	1000	44	250	77	75	108	50	144	1000
11	1000	46	50	77	150	109	50	145	25
11	1000	46	150	78	50	109	75	151	75
12	250	46	150	78	50	110	25	153	75
12	250	47	2000	78	1000	110	50	156	75
12	500	49	50	80	75	111	500	156	100
13	75	49	75	81	500	112	75	156	150
14	1000	50	25	81	1000	112	75	157	250
14	1000	50	25	84	500	115	50	158	25
14	1000	51	75	84	1000	115	50	158	25
16	25	51	100	84	1000	116	150	160	500
16	150	51	250	86	150	116	250	160	500
18	25	52	50	86	250	116	1000	163	500
18	50	52	75	87	50	118	75	165	25
18	75	53	500	87	100	119	25	165	100
19	25	53	1000	88	500	119	50	166	50
19	25	54	50	88	1000	119	50	166	250
20	500	54	1000	89	75	121	25	166	500
20	1000	55	500	90	50	121	25	169	25
20	1000	55	1000	90	75	121	50	169	500
22	50	55	2000	91	75	122	75	173	25
22	500	56	150	91	150	122	250	173	25
22	500	56	500	91	1000	122	250	173	75
24	75	59	25	93	50	124	75	174	75
24	100	59	50	93	500	124	250	174	100
25	75	63	25	94	25	126	25	175	500
25	100	63	500	94	500	126	50	176	50
26	1000	65	25	94	500	126	250	176	1000
26	1000	65	100	95	250	127	25	176	2000
26	2000	66	500	95	250	127	50	177	25
28	75	66	3000	96	75	128	25	177	25
31	25	68	1000	97	25	128	50	177	25
31	25	69	500	97	250	129	50	179	25
32	25	69	3000	97	250	129	50	179	75
34	100	70	150	99	25	129	50	179	75

N° plante	EC100	N° plant	EC100	N° plant	EC100	N° plant	EC100	N° plant	EC100
1	75	34	100	72	25	99	250	133	150
2	25	34	250	72	50	99	1000	133	150
2	50	35	50	72	250	101	25	135	25
2	100	38	25	73	75	101	100	135	50
4	500	38	75	74	25	103	25	137	250
4	1000	40	500	74	100	103	50	137	500
5	2000	40	500	74	250	103	50	137	1000
5	3000	40	1000	75	1000	105	25	138	50
6	150	42	50	75	2000	105	75	138	50
7	50	42	50	76	75	106	150	141	50
7	250	44	50	76	1000	108	25	143	250
9	500	44	100	77	25	108	50	144	1000
11	1000	44	250	77	75	108	50	144	1000
11	1000	46	50	77	150	109	50	145	25
11	1000	46	150	78	50	109	75	151	75
12	250	46	150	78	50	110	25	153	75
12	250	47	2000	78	1000	110	50	156	75
12	500	49	50	80	75	111	500	156	100
13	75	49	75	81	500	112	75	156	150
14	1000	50	25	81	1000	112	75	157	250
14	1000	50	25	84	500	115	50	158	25
14	1000	51	75	84	1000	115	50	158	25
16	25	51	100	84	1000	116	150	160	500
16	150	51	250	86	150	116	250	160	500
18	25	52	50	86	250	116	1000	163	500
18	50	52	75	87	50	118	75	165	25
18	75	53	500	87	100	119	25	165	100
19	25	53	1000	88	500	119	50	166	50
19	25	54	50	88	1000	119	50	166	250
20	500	54	1000	89	75	121	25	166	500
20	1000	55	500	90	50	121	25	169	25
20	1000	55	1000	90	75	121	50	169	500
22	50	55	2000	91	75	122	75	173	25
22	500	56	150	91	150	122	250	173	25
22	500	56	500	91	1000	122	250	173	75
24	75	59	25	93	50	124	75	174	75
24	100	59	50	93	500	124	250	174	100
25	75	63	25	94	25	126	25	175	500
25	100	63	500	94	500	126	50	176	50
26	1000	65	25	94	500	126	250	176	1000
26	1000	65	100	95	250	127	25	176	2000
26	2000	66	500	95	250	127	50	177	25
28	75	66	3000	96	75	128	25	177	25
31	25	68	1000	97	25	128	50	177	25
31	25	69	500	97	250	129	50	179	25
32	25	69	3000	97	250	129	50	179	75
34	100	70	150	99	25	129	50	179	75

TABLE 2 *A. halleri* x *A. l. petraea* BC1 progeny Cd tolerance EC100 values (µM Cd)

N° plant	EC100	N° plant	EC100	N° plant	EC100	N° plant	EC100	N° plant	EC100
4	100	75	25	156	25	238	50	299	75
4	75	75	75	156	50	238	25	299	75
5	100	75	75	156	50	238	50	301	50
5	150	86	25	176	150	239	50	301	75
5	100	86	50	176	100	239	50	310	25
11	150	86	75	176	150	239	50	310	50
11	250	88	50	177	25	240	150	310	25
11	100	88	75	177	25	240	100	318	50
12	100	88	100	179	25	242	100	318	50
12	75	90	10	179	25	242	150	323	150
12	25	90	10	179	25	242	100	323	150
14	25	90	25	181	100	244	50	331	25
14	100	94	50	181	100	244	25	331	25
18	10	94	75	185	75	246	50	336	50
18	25	94	50	185	50	246	25	336	50
26	150	95	150	189	50	252	100		
26	100	95	150	189	50	252	100		
26	100	97	75	189	50	253	50		
34	25	97	100	191	25	253	50		
34	25	103	50	191	25	253	50		
38	50	103	50	193	150	265	100		
38	100	103	75	193	100	265	10		
40	100	108	25	195	50	266	50		
40	100	108	25	195	25	266	50		
40	150	112	50	196	25	269	10		
46	250	112	50	196	25	269	10		
46	250	112	75	197	100	269	50		
49	25	119	25	197	100	270	100		
49	25	119	50	199	25	270	100		
49	50	119	25	199	75	270	100		
50	25	121	25	203	50	275	50		
50	50	121	25	203	50	275	50		
50	50	128	25	208	75	275	50		
53	100	128	25	208	25	279	50		
53	75	128	75	211	50	279	75		
55	75	129	25	211	25	279	75		
55	100	129	25	211	25	282	50		
59	100	129	50	221	50	282	25		
59	25	137	50	221	25	282	50		
59	75	137	75	221	25	290	100		
66	150	138	25	227	100	290	100		
66	150	138	25	227	100	294	50		
66	100	138	25	227	75	294	50		
69	150	144	50	228	50	298	50		
69	100	144	75	228	50	298	25		
69	100	144	75	228	50	299	75		

TABLE 3A *A. halleri* x *A. l. petraea* BC1 progeny Zn tolerance EC100 values (µM Zn)

N° plant	EC100	N° plant	EC100	N° plant	EC100	N° plant	EC100	N° plant	EC100
1	75	34	100	72	25	99	250	133	150
2	25	34	250	72	50	99	1000	133	150
2	50	35	50	72	250	101	25	135	25
2	100	38	25	73	75	101	100	135	50
4	500	38	75	74	25	103	25	137	250
4	1000	40	500	74	100	103	50	137	500
5	2000	40	500	74	250	103	50	137	1000
5	3000	40	1000	75	1000	105	25	138	50
6	150	42	50	75	2000	105	75	138	50
7	50	42	50	76	75	106	150	141	50
7	250	44	50	76	1000	108	25	143	250
9	500	44	100	77	25	108	50	144	1000
11	1000	44	250	77	75	108	50	144	1000
11	1000	46	50	77	150	109	50	145	25
11	1000	46	150	78	50	109	75	151	75
12	250	46	150	78	50	110	25	153	75
12	250	47	2000	78	1000	110	50	156	75
12	500	49	50	80	75	111	500	156	100
13	75	49	75	81	500	112	75	156	150
14	1000	50	25	81	1000	112	75	157	250
14	1000	50	25	84	500	115	50	158	25
14	1000	51	75	84	1000	115	50	158	25
16	25	51	100	84	1000	116	150	160	500
16	150	51	250	86	150	116	250	160	500
18	25	52	50	86	250	116	1000	163	500
18	50	52	75	87	50	118	75	165	25
18	75	53	500	87	100	119	25	165	100
19	25	53	1000	88	500	119	50	166	50
19	25	54	50	88	1000	119	50	166	250
20	500	54	1000	89	75	121	25	166	500
20	1000	55	500	90	50	121	25	169	25
20	1000	55	1000	90	75	121	50	169	500
22	50	55	2000	91	75	122	75	173	25
22	500	56	150	91	150	122	250	173	25
22	500	56	500	91	1000	122	250	173	75
24	75	59	25	93	50	124	75	174	75
24	100	59	50	93	500	124	250	174	100
25	75	63	25	94	25	126	25	175	500
25	100	63	500	94	500	126	50	176	50
26	1000	65	25	94	500	126	250	176	1000
26	1000	65	100	95	250	127	25	176	2000
26	2000	66	500	95	250	127	50	177	25
28	75	66	3000	96	75	128	25	177	25
31	25	68	1000	97	25	128	50	177	25
31	25	69	500	97	250	129	50	179	25
32	25	69	3000	97	250	129	50	179	75
34	100	70	150	99	25	129	50	179	75

N° plant	EC100	N° plant	EC100	N° plant	EC100	N° plant	EC100	N° plant	EC100
181	250	209	250	246	500	286	50	337	75
181	500	211	25	247	500	289	25	338	150
181	3000	211	50	248	25	290	500	338	250
184	75	211	50	248	500	290	500		
184	250	217	75	249	100	291	25		
185	250	219	25	249	100	291	250		
185	500	219	250	249	150	293	500		
186	25	220	25	252	25	293	2000		
186	75	220	25	252	500	294	25		
189	25	220	50	252	2000	294	25		
189	50	221	25	253	500	298	25		
189	50	221	50	253	500	298	50		
190	50	221	75	253	500	298	100		
191	50	222	1000	256	25	299	75		
191	50	226	25	256	75	299	250		
191	75	226	50	256	250	299	500		
192	25	227	50	259	250	300	25		
192	1000	227	1000	262	250	301	500		
193	500	227	2000	262	500	301	500		
193	1000	228	75	264	50	301	1000		
194	250	228	75	264	75	302	50		
194	500	229	50	264	1000	302	50		
194	1000	230	25	265	500	303	50		
195	25	230	75	265	1000	303	250		
195	25	230	250	266	100	304	500		
195	75	231	50	266	250	310	150		
196	50	233	25	269	50	310	250		
196	75	233	500	269	75	310	500		
197	25	235	75	270	500	312	75		
197	25	238	250	270	500	312	75		
197	75	238	250	270	500	312	500		
199	25	238	1000	271	50	316	250		
199	50	239	150	273	50	318	250		
199	50	239	150	273	50	318	500		
202	75	240	1000	274	25	322	25		
202	150	240	1000	275	25	322	75		
202	250	240	1000	275	75	323	250		
203	25	241	50	276	500	323	500		
203	25	242	500	279	100	323	500		
203	25	242	500	279	500	329	75		
205	25	242	1000	279	3000	329	250		
205	50	244	50	280	50	329	500		
207	150	244	50	281	75	331	75		
208	25	245	75	282	25	331	100		
208	1000	245	100	282	25	331	150		
208	1000	245	500	282	100	336	100		
209	25	246	25	286	50	336	250		
209	25	246	75	286	50	336	250		

TABLE 3B BC1 parental genotypes (*A. halleri*, *A. l. petraea* 1, *A. l. petraea* 2, F1) Zn tolerance EC100 values (µM Zn)

Genotype	EC100	Genotype	EC100	Genotype	EC100
A. halleri	2000	*A. l. petraea* 1	25	F1	1000
A. halleri	3000	*A. l. petraea* 1	25	F1	2000
A. halleri	3000	*A. l. petraea* 1	25	F1	2000
A. halleri	3000	*A. l. petraea* 1	50	F1	2000
A. halleri	3000	*A. l. petraea* 1	50	F1	2000
A. halleri	3000	*A. l. petraea* 1	50	F1	3000
A. halleri	3000	*A. l. petraea* 2	25	F1	500
A. halleri	3000	*A. l. petraea* 2	25	F1	1000
A. halleri	3000	*A. l. petraea* 2	50	F1	1000
A. halleri	3000	*A. l. petraea* 2	50	F1	2000
A. halleri	3000	*A. l. petraea* 2	50	F1	2000
A. halleri	3000	*A. l. petraea* 2	50	F1	2000
		A. l. petraea 2	50		
		A. l. petraea 2	50		
		A. l. petraea 2	250